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(54) NUCLEIC ACID TYPING BY POLYMERASE EXTENSION OF OLIGONUCLEOTIDES USING TERMINATOR MIXTURES

NUKLEINSÄURETYPISIERUNG DURCH POLYMERASEVERLÄNGERUNG VON OLIGONUKLEOTIDEN UNTER VERWENDUNG VON TERMINATOR-MISCHUNGEN

DETERMINATION D'ACIDES NUCLEIQUES PAR EXTENSION DE LA POLYMERASE D'OLIGONUCLEOTIDES A L'AIDE DE MELANGES TERMINATEURS

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(56) References cited:
EP-A- 0 412 883
WO-A-91/13075
WO-A-92/16657
US-A- 4 656 127
US-A- 4 760 017
US-A- 4 851 331
US-A- 4 962 020

- **NUCLEIC ACIDS RESEARCH, vol. 18, no. 12, 25 June 1990, page 3671 XP000310585 SOKOLOV B P: "PRIMER EXTENSION TECHNIQUE FOR THE DETECTION OF SINGLE NUCLEOTIDE IN GENOMIC DNA"**
- **NUCLEIC ACID RESEARCH, vol. 13, - 12 August 1985 pages 5457-5469, XP002028817 DELIUS H. ET AL.,: "Separation of complementary strands of plasmid DNA using the biotin-avidin system and its application to heteroduplex formation and RNA/DNA hybridizations in electron microscopy"**
- **Science, Volume 238, issued 16 October 1987, J.M. PROBER et al., "A System for Rapid DNA Sequencing with Fluorescent Chain-Terminating Dideoxynucleotides", pages 336-341, see especially page 337, column 1, paragraph 3 and Abstract.**
- **Science, Volume 242, issued 14 October 1988, U. LANDEGRAN et al., "DNA Diagnostics - Molecular Techniques and Automation", pages 229-237, see entire document.**
- **Analytical Biochemistry, Volume 174, issued 1988, E.D. HYMAN, "A New Method of Sequencing DNA", pages 423-436, see especially the Abstract.**
- **Scientific American, issued April 1990, K.B. MULLIS, "The Unusual Origin of the Polymerase Chain Reaction", pages 56-65, especially page 60.**

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Description

Background of the Invention

[0001] This invention relates to the field of nucleic acid sequence detection. The detection of nucleic acid sequences can be used in two general contexts. First, the detection of nucleic acid sequences can be used to determine the presence or absence of a particular genetic element. Second, the detection of nucleic acid sequences can be used to determine the specific type of a particular genetic element that is present. Variant genetic elements usually exist. Many techniques have been developed (1) to determine the presence of specific nucleic acid sequences, and (2) to compare homologous segments of nucleic acid sequence to determine if the segments are identical or if they differ at one or more nucleotides. Practical applications of these techniques include genetic disease diagnoses, infectious disease diagnoses, forensic techniques, paternity determinations, and genome mapping.

[0002] In general, the detection of nucleic acids in a sample and the subtypes thereof depends on the technique of specific nucleic acid hybridization in which the oligonucleotide probe is annealed under conditions of high stringency to nucleic acids in the sample, and the successfully annealed probes are subsequently detected (see Spiegelman, S., Scientific American, Vol. 210, p. 48 (1964)).

[0003] The most definitive method for comparing DNA segments is to determine the complete nucleotide sequence of each segment. Examples of how sequencing has been used to study mutations in human genes are included in the publications of Engelke, et al., Proc. Natl. Acad. Sci. U.S.A., 85:544-548 (1988) and Wong, et al., Nature, 330:384-386 (1987). At the present time, it is not practical to use extensive sequencing to compare more than just a few DNA segments because the effort required to determine, interpret, and compare sequence information is time-consuming.

[0004] A commonly used screen for DNA polymorphisms arising from DNA sequence variation consists of digesting DNA with restriction endonucleases and analyzing the resulting fragments by means of Southern blots, as described by Botstein, et al., Am. J. Hum. Genet., 32:314-331 (1980) and White, et al., Sci. Am., 258:40-48 (1988). Mutations that affect the recognition sequence of the endonuclease will preclude enzymatic cleavage at that site, thereby altering the cleavage pattern of that DNA. DNAs are compared by looking for differences in restriction fragment lengths. A major problem with this method (known as restriction fragment length polymorphism mapping or RFLP mapping) is its inability to detect mutations that do not affect cleavage with a restriction endonuclease. Thus, many mutations are missed with this method. One study, by Jeffreys, Cell, 18:1-18 (1979), was able to detect only 0.7% of the mutational variants estimated to be present in a 40,000 base pair region of human DNA. Another problem is that the methods used to detect restriction fragment length polymorphisms are very labor intensive, in particular, the techniques involved with Southern blot analysis.

[0005] A technique for detecting specific mutations in any segment of DNA is described in Wallace, et al., Nucl.

[0006] Acids Res., 9:879-894 (1981). It involves hybridizing the DNA to be analyzed (target DNA) with a complementary, labeled oligonucleotide probe. Due to the thermal instability of DNA duplexes containing even a single base pair mismatch, differential melting temperature can be used to distinguish target DNAs that are perfectly complementary to the probe from target DNAs that differ by as little as a single nucleotide. In a related technique, described in Landegren, et al., Science, 41:1077-1080 (1988), oligonucleotide probes are constructed in pairs such that their junction corresponds to the site on the DNA being analyzed for mutation. These oligonucleotides are then hybridized to the DNA being analyzed. Base pair mismatch between either oligonucleotide and the target DNA at the junction location prevents the efficient joining of the two oligonucleotide probes by DNA ligase.

A. Nucleic acid hybridization

[0007] The base pairing of nucleic acids in a hybridization reaction forms the basis of most nucleic acid analytical and diagnostic techniques. In practice, tests based only on parameters of nucleic acid hybridization function poorly in cases where the sequence complexity of the test sample is high. This is partly due to the small thermodynamic differences in hybrid stability, generated by single nucleotide changes, and the fact that increasing specificity by lengthening the probe has the effect of further diminishing this differential stability. Nucleic acid hybridization is, therefore, generally combined with some other selection or enrichment procedure for analytical and diagnostic purposes.

[0008] Combining hybridization with size fractionation of hybridized molecules as a selection technique has been one general diagnostic approach. Size selection can be carried out prior to hybridization. The best known prior size selection technique is Southern Blotting (see Southern, E., Methods in Enzymology, 69:152 (1980). In this technique, a DNA sample is subjected to digestion with restriction enzymes which introduce double stranded breaks in the phosphodiester backbone at or near the site of a short sequence of nucleotides which is characteristic for each enzyme. The resulting heterogeneous mixture of DNA fragments is then separated by gel electrophoresis, denatured, and transferred to a solid phase where it is subjected to hybridization analysis in situ using a labeled nucleic acid probe. Fragments which contain sequences complementary to the labeled probe are revealed visually or densitometrically as

bands of hybridized label. A variation of this method is Northern Blotting for RNA molecules. Size selection has also been used after hybridization in a number of techniques, in particular by hybrid protection techniques, by subjecting probe/nucleic acid hybrids to enzymatic digestion before size analysis.

B. Polymerase extension of duplex primer:template complexes

[0009] Hybrids between primers and DNA targets can be analyzed by polymerase extension of the hybrids. A modification of this methodology is the polymerase chain reaction in which the purification is produced by sequential hybridization reactions of anti-parallel primers, followed by enzymatic amplification with DNA polymerase (see Saiki, et al., Science 239:487-491 (1988)). By selecting for two hybridization reactions, this methodology provides the specificity lacking in techniques that depend only upon a single hybridization reaction.

[0010] It has long been known that primer-dependent DNA polymerases have, in general, a low error rate for the addition of nucleotides complementary to a template. This feature is essential in biology for the prevention of genetic mistakes which would have detrimental effects on progeny. The specificity inherent in this enzymological reaction has been widely exploited as the basis of the "Sanger" or dideoxy chain termination sequencing methodology which is the ultimate-nucleic acid typing experiment. one type of Sanger DNA sequencing method makes use of mixtures of the four deoxynucleoside triphosphates, which are normal DNA precursors, and one of the four possible dideoxynucleoside triphosphates, which have a hydrogen atom instead of a hydroxyl group attached to the 3' carbon atom of the ribose sugar component of the nucleotide. DNA chain elongation in the 5' to 3' direction ("downstream") requires this hydroxyl group. As such, when a dideoxynucleotide is incorporated into the growing DNA chain, no further elongation can occur. With one dideoxynucleotide in the mixture, DNA polymerases can, from a primer:template combination, produce a population of molecules of varying length, all of which terminate after the addition of one out of the four possible nucleotides. The series of four independent reactions, each with a different dideoxynucleotide, generates a nested set of fragments, all starting at the same 5' terminus of the priming DNA molecule and terminating at all possible 3' nucleotide positions.

[0011] Another utilization of dideoxynucleoside triphosphates and a polymerase in the analysis of DNA involves labeling the 3' end of a molecule. One prominent manifestation of this technique provides the means for sequencing a DNA molecule from its 3' end using the Maxam-Gilbert method. In this technique, a molecule with a protruding 3' end is treated with terminal transferase in the presence of radioactive dideoxy-ATP. One radioactive nucleotide is added, rendering the molecule suitable for sequencing. Both methods of DNA sequencing using labeled dideoxynucleotides require electrophoretic separation of reaction products in order to derive the typing information. Most methods require four separate gel tracks for each typing determination.

[0012] The following two patents describe other methods of typing nucleic acids which employ primer extension and labeled nucleotides. Mundy (U.S. Patent No. 4,656,127) describes a method whereby a primer is constructed complementary to a region of a target nucleic acid of interest such that its 3' end is close to a nucleotide in which variation can occur. This hybrid is subject to primer extension in the presence of a DNA polymerase and four deoxynucleoside triphosphates, one of which is an α-thionucleotide. The hybrid is then digested using an exonuclease enzyme which cannot use thio-derivatized DNA as a substrate for its nucleolytic action (for example Exonuclease III of E. coli). If the variant nucleotide in the template is complementary to one of the thionucleotides in the reaction mixture, the resulting extended primer molecule will be of a characteristic size and resistant to the exonuclease; hybrids without thio-derivatized DNA will be digested. After an appropriate enzyme digest to remove underivatized molecules, the thio-derivatized molecule can be detected by gel electrophoresis or other separation technology.

[0013] Vary and Diamond (U.S. Patent No. 4,851,331) describes a method similar to that of Mundy wherein the last nucleotide of the primer corresponds to the variant nucleotide of interest. Since mismatching of the primer and the template at the 3' terminal nucleotide of the primer is counterproductive to elongation, significant differences in the amount of incorporation of a tracer nucleotide will result under normal primer extension conditions. This method depends on the use of a DNA polymerase, e.g., AMV reverse transcriptase, that does not have an associated 3' to 5' exonuclease activity.

[0014] The methods of Mundy and of Vary and Diamond have drawbacks. The method of Mundy is useful but cumbersome due to the requirements of the second, different enzymological system where the non-derivatized hybrids are digested. The method of Vary is complicated by the fact that it does not generate discrete reaction products. Any "false" priming will generate significant noise in such a system which would be difficult to distinguish from a genuine signal.

[0015] The present invention circumvents the problems associated with the methods of Mundy and of Vary and Diamond for typing nucleic acid with respect to particular nucleotides. With methods employing primer extension and a DNA polymerase, the current invention will generate a discrete molecular species one base longer than the primer itself. In many methods, particularly those employing the polymerase chain reaction, the type of reaction used to purify the nucleic acid of interest in the first step can also be used in the subsequent detection step. Finally, with terminators which are labeled with different detector moieties (for example different fluorophors having different spectral properties),

it will be possible to use only one reagent for all sequence detection experiments. Furthermore, if techniques are used to separate the terminated primers post-reaction, sequence detection experiments at more than one locus can be carried out in the same tube.

[0016] A recent article by Mullis (Scientific American, April 1990, pp. 56-65) suggests an experiment, which apparently was not performed, to determine the identity of a targeted base pair in a piece of double-stranded DNA. Mullis suggests using four types of dideoxynucleosides triphosphate, with one type of dideoxynucleoside triphosphate being radioactively labeled.

[0017] The present invention permits analyses of nucleic acid sequences that can be useful in the diagnosis of infectious diseases, the diagnosis of genetic disorders, and in the identification of individuals and their parentage.

[0018] A number of methods have been developed for these purposes. Although powerful, such methodologies have been cumbersome and expensive, generally involving a combination of techniques such as gel electrophoresis, blotting, hybridization, and autoradiography or non-isotopic revelation. Simpler technologies are needed to allow the more widespread use of nucleic acid analysis. In addition, tests based on nucleic acids are currently among the most expensive of laboratory procedures and for this reason cannot be used on a routine basis. Finally, current techniques are not adapted to automated procedures which would be necessary to allow the analysis of large numbers of samples and would further reduce the cost.

[0019] The current invention provides a method that can be used to diagnose or characterize nucleic acids in biological samples without recourse to gel electrophoretic size separation of the nucleic acid species. This feature renders this process easily adaptable to automation and thus will permit the analysis of large numbers of samples at relatively low cost. Because nucleic acids are the essential blueprint of life, each organism or individual can be uniquely characterized by identifiable sequences of nucleic acids. It is, therefore, possible to identify the presence of particular organisms or demonstrate the biological origin of certain samples by detecting these specific nucleic acid sequences.

Summary of the Invention

[0020] The invention provides a method of determining the identity of a nucleotide base at a specific position in a nucleic acid of interest which comprises:

(a) in the alternative:

(i) if the nucleic acid of interest is double-stranded, treating a sample containing the nucleic acid so as to obtain an unpaired-base template nucleic-acid sample comprising a template of unpaired nucleotide bases spanning the specific position, or

(ii) if the nucleic acid of interest is single-stranded, obtaining a sample of the nucleic acid to serve as an unpaired-base template nucleic-acid sample comprising a template of unpaired nucleotide bases spanning the specific position;

(b) obtaining an oligonucleotide primer having a nucleotide sequence capable of hybridising with the template nucleic acid to form a duplex wherein the nucleotide base to be identified is immediately adjacent to, and downstream of, the 3' end of the primer;

(c) immobilising the oligonucleotide primer on a solid support, such as to permit the immobilised primer to hybridise with the template nucleic acid to form a duplex wherein the nucleotide base to be identified is immediately adjacent to, and downstream of, the 3' end of the primer;

(d) contacting the immobilised oligonucleotide primer under hybridising conditions with unpaired-base-template sample from step (a) to form on the solid support a duplex between the immobilised primer and the template nucleic acid wherein the nucleotide base to be identified is immediately adjacent to, and downstream of, the 3' end of the primer in the duplex;

(e) contacting the duplex from step (d) with a reagent composition which comprises an aqueous carrier and an admixture of four different terminators of a nucleic acid template-dependent, primer extension reaction, each of the terminators being capable of specifically terminating the extension reaction in a manner strictly dependent on the identity of the unpaired nucleotide base in the template immediately adjacent to, and downstream of, the 3' end of a primer, wherein one of the terminators is complementary to the nucleotide base to be identified and at least one of the terminators is labeled with a detectable marker, under conditions permitting base pairing of the complementary terminator present in the reagent with the nucleotide base to be identified and occurrence of a template-dependent, primer extension reaction so as to incorporate the complementary terminator at the 3' end of the primer to form an extended primer such that the primer has been extended by the complementary terminator;

(f) removing labeled terminator not incorporated in the primer from the solid support; and

(g) identifying any detectable marker present at the 3' end of the immobilised extended primer from step (e) on

the solid support, to determine the identity of the nucleotide base to be identified.

[0021] The invention also provides a method for determining the genotype of an organism at one or more particular genetic loci which comprises:

(a) obtaining from the organism a sample containing genomic DNA; and
(b) identifying the nucleotide base or bases present at each of one or more specific positions in nucleic acids of interest, each such base or bases being identified using a method of the invention and thereby identifying different alleles and thereby, in turn, determining the genotype of the organism at one or more particular genetic loci.

[0022] The invention also provides a method of determining the presence or absence of a particular nucleotide sequence in a nucleic acid of interest which comprises:

(a) in the alternative:

(i) if the nucleic acid of interest is double-stranded, treating a sample containing the nucleic acid so as to obtain an unpaired-base template nucleic-acid sample comprising a template of unpaired nucleotide bases, or
(ii) if the nucleic acid of interest is single-stranded, obtaining a sample of the nucleic acid to serve as an unpaired-base template nucleic-acid sample comprising a template of unpaired nucleotide bases;

(b) obtaining an oligonucleotide primer having a nucleotide sequence capable of hybridising with the particular nucleotide sequence if the particular nucleotide sequence is present in the template nucleic acid to form a primer-extendable duplex between the primer and the particular nucleotide sequence such that the hybridised primer is extendable at a 3' end by a nucleic acid template-dependent, primer extension reaction;
(c) immobilising the oligonucleotide primer on a solid support, such as to permit the immobilised primer to hybridise with the template nucleic acid to form a primer-extendable duplex if the particular nucleotide sequence is present in the template nucleic acid such that the hybridised primer is extendable at the 3' end by a nucleic acid template-dependent, primer extension reaction;
(d) contacting the immobilised oligonucleotide primer under hybridising conditions with unpaired-base-template sample from step (a) to form on the solid support a primer-extendable duplex between the immobilised primer and the template nucleic acid if the particular nucleotide sequence is present in the template nucleic acid such that the hybridised primer is extendable at the 3' end by a nucleic acid template-dependent, primer extension reaction;
(e) contacting the primer-extendable duplex from step (d), if any, with a reagent composition which comprises an aqueous carrier and an admixture of four different terminators of a nucleic acid template-dependent, primer extension reaction, at least one of the terminators being labeled with a detectable marker, each of the terminators being capable of specifically terminating the primer extension reaction in a manner dependent on the identity of an unpaired nucleotide base in a template immediately adjacent to, and downstream of, the 3' end of a primer hybridised to the template such that the hybridised primer is extendable at a 3' end by the primer extension reaction, the primer-extendable duplex, if any, being contacted with the reagent composition under conditions permitting base pairing of a complementary terminator present in the reagent with a nucleotide base in the template nucleic acid of the duplex immediately adjacent to, and downstream of, the 3' end of the hybridised primer of the duplex and occurrence of the template-dependent, primer extension reaction so as to incorporate the complementary terminator at the 3' end of the primer to form an extended primer such that the primer is extended by the complementary terminator if the particular nucleotide sequence is present in the template nucleic acid, no such extended primer being formed if the particular nucleotide sequence is not present in the template nucleic acid;
(f) removing labeled terminator not incorporated in the primer from the solid support; and
(g) detecting the presence or absence of a detectable marker at the 3' end of the immobilised primer from step (e) on the solid support, to determine the presence or absence of the particular nucleotide sequence in the nucleic acid of interest.

[0023] The invention also provides a method of typing a sample containing nucleic acids which comprises:

(a) determining the presence or absence of one or more particular nucleotide sequences, the presence or absence of each such nucleotide sequence being determined by a method as defined immediately above; and optionally
(b) identifying the nucleotide base or bases present at each of one or more specific positions, each such nucleotide base being identified using a method of the invention and each such specific position being determined using a different primer.

Brief Description of the Figures

[0024]

Figure 1. Autoradiography of labeled DNA products after fractionation on a polyacrylamide/urea gel. Panel A shows products of the "A" extension reaction on oligonucleotide primer 182 directed by template oligonucleotides 180 or 181. Panel B shows products of the "B" termination reaction on oligonucleotide primer 182 annealed to template oligonucleotides 180 or 181. Panel C shows the same products as in panel B after purification on magnetic beads. Note: oligodeoxynucleotide 182 was used as supplied by Midland Certified Reagents with no further purification. The minor bands above and below the main band are presumably contaminants due to incomplete reactions or side reactions that occurred during the step-wise synthesis of the oligonucleotide. For a definition of the "A" extension reaction and the "B" termination reaction, see "A. GENERAL METHODS" in the Detailed Description of the Invention.

Figure 2. Detection of Sequence Polymorphisms in PCR Products. Target polymorphic DNA sequence showing amplification primers, detection primers, and molecular clone (plasmid) designations. For each primer, sites of binding to one or the other strand of the target DNA sequence are indicated by underlining, and the direction of DNA synthesis is indicated by an arrow. Numbering for the target sequence is shown in the righthand margin. Polymorphic sites at positions 114 and 190 are indicated by bold lettering and a slash between the two polymorphic possibilities.

Figure 3. Autoradiogram of gel-analyzed polymorphism test on PCR products. Templates from PCR products of p183, p624, or p814 were analyzed with the detection primers, TGL182 and TGL166, in a template-directed chain extension experiment, as described in the specification. Reaction products were fractionated by size on a polyacrylamide/urea DNA sequencing gel, and incorporation of [^{35}S-α-thio-dideoxy adenosine monophosphate was assayed by autoradiography.

Figure 4. Gel electrophoretic analysis of the labelled extension products of primers TGL346 and TGL391. Productive primer-template complexes of TGL346 or TGL391 with the bead-bound oligonucleotide template, TGL382, were subjected to primer extension labelling reactions with the four different [α-thio-^{35}S]dideoxynucleoside triphosphate mixes. Labelled primer DNA was released from the washed beads and electrophoresed on an 8% polyacrylamide/8 M urea DNA sequencing gel (2.5 pmoles of primer/lane), then analyzed by autoradiography. The four lanes shown for the primer TGL346 indicate that labelling occurred predominantly with the ddC mix, indicating that the next unpaired base in the TGL382 template adjacent to the 3' end of TGL346 was a G (see sequence given in Example 4). The four lanes shown for the primer TGL391 indicate that the labelling occurred predominantly with the ddT mix, indicating that the next unpaired base in the TGL382 template adjacent to the 3' end of TGL391 was an A.

Figure 5. Autoradiographic analyses of total radioactivity bound to beads. The bead suspensions, containing the products of the extension reactions described in Figure 5, were spotted onto filter paper (1 pmole of primer per spot) and exposed to X-ray film to assay total bead-bound radioactivity. As shown, TGL346 predominantly incorporated label from the ddC mix and TGL391 predominantly from the ddT mix.

Figure 6. PCR-amplified polymorphic locus of mammalian DNA. Shown is a 327 basepair segment of mammalian DNA that was amplified from samples of genomic DNA using the PCR primers TGL240 (biotinylated) and TGL239 (unbiotinylated). Samples of DNA from two homozygous individuals, ESB164 (genotype AA) and EA2014 (genotype BB), were subjected to the analyses described in Example 5. The complete DNA sequence of the A allele at this locus is shown, with the polymorphic sites where the B allele sequence differs from the A allele sequence indicated by the bases underneath the A sequence. The detection primer, TGL308, is shown base-paired with the template strand extending from the biotinylated primer. For the A allele, the first unpaired template base immediately downstream of the 3' end of TGL308 is a C, and for the B allele this base is an A. Thus, the A allele should result in labelling of TGL308 by the ddG mix only, and the B allele should result in labelling by the ddT mix only.

Figure 7. Gel electrophoretic analysis of PCR products from two different homozygous individuals. Primers TGL240 and TGL239 were used to amplify genomic DNA (obtained from blood) from two individuals, ESB164 and EA2014. The products of the extension reactions for primer TGL308, annealled to the bead-bound, PCR-generated template as outlined in Figure 7, were analyzed by electrophoresis on an 8% polyacrylamide/8 M urea DNA sequencing gel as outlined in Figure 5. Shown for individual ESB164 (genotype AA: labelling expected from the ddG mix) are 250

fmoles of extended primer from the four different ddNTP labelling reactions. Shown for individual EA2014 (genotype BB: labelling expected from the ddT mix) are loadings of 25, 75, and 250 fmoles of extended primer from the four different ddNTP labelling reactions.

Figure 8. Autoradiographic analyses of total and NaOH-eluted radioactivity from TGL308 primer extension reactions. Primer TGL308 was used to analyze the genotypes of individuals ESB164 and EA2014 as outlined in Example 5 and Figures 7 and 8. Total bead-associated radioactivity was determined by directly spotting a suspension of beads containing 75 fmoles of primer onto filter paper followed by autoradiographic detection of the label in the spot. Radioactivity specifically associated with the TGL308 primer was determined by magnetically immobilizing the beads, eluting the primer with NaOH as described in Examples 4 and 5, and spotting on filter paper an amount corresponding to 75 fmoles. Label in these spots was also detected by autoradiography.

Figure 9. Data is shown from GBA on single stranded nucleic acid produced by asymmetric PCR from human DNA samples of different genotypes. The DNA sequence being interrogated is from the HLA DPA1 locus at the polymorphic sequence coding for amino acid 31 of the DP alpha chain (Marsh, S.G.E. and Bodmer, J.G., HLA Class II Nucleotide Sequences, 1991. Human Immunol. 31, 207-227 [1991]) and is shown in the middle of the figure. Identification of the nucleotide immediately downstream of the pjrimer is accomplished by enzyme-linked detection and is visualized as an orange color change in the well corresponding to the nucleotide which is inserted by the T7 DNA polymerase. Homozygotes only have one positive well, heterozygotes have two. The sequence of the GBA primer is indicated by an arrow whose tail is the 5' and head is the 3' end of the oligonucleotide.

Figure 10. Data is shown from GBA on single stranded nucleic acid produced by asymmetric PCR from equine DNA samples of different genotypes. The DNA sequence being interrogated is from the HLA DPA1 locus at the polymorphic sequence coding for amino acid 50 of the DP alpha chain (Marsh, S.G.E. and Bodmer, J.G., HLA Class II Nucleotide Sequences, 1991. Human Immunol. 31, 207-227 [1991]) and is shown in the middle of the Figure.

Figure 11. Data is shown from GBA on single stranded nucleic acid produced by asymmetric PCR from equine DNA samples of different genotypes. The DNA sequence being interrogated is from the anonymous locus JH85 at the polymorphic sequence at nucleotide number 122 with respect to the original ccloned genomic peice (unpublished results) and is shown in the middle of the figure. At this position, the "B" allele contains one extra base. For this reason, a different nucleotide position is interrogated by primer #307 as compared to #308. Nevertheless, the results of both strand interrogations allow for unambiguous typing.

Figure 12. Data shown are the results of a quantitative GBA of equine locus JH85. Following addition of substrate, the microplate was read kinetically, in a "Vmax" model 96-well spectrophotometer (Molecular Devices, Inc., Menlo Park, CA). Values are expressed as a Vmax in milli OD units per minute. The GBA results for the AA homozygote (solid bars), the AB heterozygote (open bars), and BB homozygote (stippled bars) single stranded templates is indicated for the four biotinylated ddNTPs analyzed in separate wells. Numerical values obtained are indicated at the top of each bar.

Detailed Description of the Invention

[0025] The invention provides a method of determining the identity of a nucleotide base at a specific position in a nucleic acid of interest which comprises:

(a) in the alternative:

(i) if the nucleic acid of interest is double-stranded, treating a sample containing the nucleic acid so as to obtain an unpaired-base template nucleic-acid sample comprising a template of unpaired nucleotide bases spanning the specific position, or
(ii) if the nucleic acid of interest is single-stranded, obtaining a sample of the nucleic acid to serve as an unpaired-base template nucleic-acid sample comprising a template of unpaired nucleotide bases spanning the specific position;

(b) obtaining an oligonucleotide primer having a nucleotide sequence capable of hybridising with the template nucleic acid to form a duplex wherein the nucleotide base to be identified is immediately adjacent to, and downstream of, the 3' end of the primer;

(c) immobilising the oligonucleotide primer on a solid support, such as to permit the immobilised primer to hybridise with the template nucleic acid to form a duplex wherein the nucleotide base to be identified is immediately adjacent to, and downstream of, the 3' end of the primer;

(d) contacting the immobilised oligonucleotide primer under hybridising conditions with unpaired-base-template sample from step (a) to form on the solid support a duplex between the immobilised primer and the template nucleic acid wherein the nucleotide base to be identified is immediately adjacent to, and downstream of, the 3' end of the primer in the duplex;

(e) contacting the duplex from step (d) with a reagent composition which comprises an aqueous carrier and an admixture of four different terminators of a nucleic acid template-dependent, primer extension reaction, each of the terminators being capable of specifically terminating the extension reaction in a manner strictly dependent on the identity of the unpaired nucleotide base in the template immediately adjacent to, and downstream of, the 3' end of a primer, wherein one of the terminators is complementary to the nucleotide base to be identified and at least one of the terminators is labeled with a detectable marker, under conditions permitting base pairing of the complementary terminator present in the reagent with the nucleotide base to be identified and occurrence of a template-dependent, primer extension reaction so as to incorporate the complementary terminator at the 3' end of the primer to form an extended primer such that the primer has been extended by the complementary terminator;

(f) removing labeled terminator not incorporated in the primer from the solid support; and

(g) identifying any detectable marker present at the 3' end of the immobilised extended primer from step (e) on the solid support, to determine the identity of the nucleotide base to be identified.

[0026] Preferably, each of the four terminators is labeled with a detectably different detectable marker.

[0027] Preferably, the oligonucleotide primer is immobilised at one of a plurality of separate test locations on the solid support. In this case, it is preferred that steps (c) to (g) are repeated four times with the same oligonucleotide primer from step (b) and the same unpaired-base-template sample from step (a) at four separate test locations on the solid support, with a corresponding one of four different reagent compositions being used in step (e) at each of the four test locations, in each of the four reagent compositions only one of the terminators being labeled with a detectable marker, a different one of the four terminators being labeled in each of the four different reagent compositions, so that identifying a detectable marker present at the 3' end of the immobilised extended primer from step (e) at one of the four test locations in step (g) determines the identity of the nucleotide base to be identified.

[0028] Preferably, in the reagent of step (e), the terminators comprise a nucleotide or nucleotide analog.

[0029] Preferably, the terminators comprise nucleotides, nucleotide analogs, dideoxynucleotides or arabinoside triphosphates. More preferably, the terminators comprise one or more of dideoxyadenosine triphosphate (ddATP), dideoxycytosine triphosphate (ddCTP), dideoxyguanosine triphosphate (ddGTP), dideoxythymidine triphosphate (ddTTP), or dideoxyuridine triphosphate (ddUTP). the terminators is an isotopically labeled moiety, a chromophore, a fluorophore, a protein moiety, or a moiety to which an isotopically labeled moiety, a chromophore, a fluorophore, or a protein moiety can be attached. The . subject invention also provides a reagent wherein each of the different detectable markers is a different fluorophore.

[0030] Optionally, the admixture of terminators of step (e) above further comprises pyrophosphatase.

[0031] Methods of the invention can be used in a DNA polymerase primer extension reaction to type nucleic acid sequences of interest that are complementary to one or more oligonucleotide primers by chemically or physically separating the polymerase extended primers from the chain terminator reagent and analysing the terminal additions. Any kind of terminator that inhibits further elongation can be used, for example, a dideoxynucleoside triphosphate. Several approaches can be used for the labeling and detection of terminators: (1) radioactivity and its detection by either autoradiography or scintillation counting, (2) fluorescence or absorption spectroscopy, (3) mass spectrometry, or (4) enzyme activity, using a protein moiety. The identity of each terminator can be determined individually, i.e., one at a time, or simultaneously.

[0032] In one embodiment, step (e) employs a reagent containing four labeled terminators, each terminator being labeled with a different detectable marker. The duplex of primer and the nucleic acid of interest is contacted with the terminator admixture under conditions permitting base pairing of a complementary terminator with the nucleotide base to be identified and the occurrence of a template-dependent, primer extension reaction so as to incorporate the terminator at the 3' end of the primer.

[0033] The net result is that the oligonucleotide primer has been extended by one terminator. Next, the identity of the detectable marker present at the 3' end of the extended primer is determined. The identity of the detectable marker indicates which terminator has base paired to the next base in the nucleic acid of interest. Since the terminator is complementary to the next base in the nucleic acid of interest, the identity of the next base in the nuclei acid of interest is thereby determined.

[0034] In another embodiment, only one of the terminators of the terminator admixture of step (e) has a detectable marker. The duplex of primer and the nucleic acid of interest is contacted with the terminator admixture under conditions

permitting base pairing of a complementary terminator with the nucleotide base to be identified and the occurrence of a template-dependent, primer extension reaction so as to incorporate the terminator at the 3' end of the primer. The net result is that the oligonucleotide primer has been extended by one terminator.

[0035] The original duplex of primer and the nucleic acid of interest is then contacted with three different reagents, with a different one of each of the four terminators being labeled in each of the four parallel reaction steps. Next, the products of the four parallel template-dependent, primer extension reactions are examined to determine which of the products has a detectable marker. The product with a detectable marker indicates which terminator has base paired to the next base in the nucleic acid of interest. Since the terminator is complementary to the next base in the nucleic acid of interest, the identity of the next base in the nucleic acid of interest is thereby determined.

[0036] Both of the methods for determining the identity of a nucleotide base at a specific position in a nucleic acid of interest label the primer after hybridization between the primer and the template. If the template-dependent enzyme has no exonuclease function, the 3' end of the primer must be base paired for the labeling by a terminator to occur.

[0037] The subject invention also provides a method of determining the presence or absence of a particular nucleotide sequence in a nucleic acid of interest which comprises:

(a) in the alternative:

(i) if the nucleic acid of interest is double-stranded, treating a sample containing the nucleic acid so as to obtain an unpaired-base template nucleic-acid sample comprising a template of unpaired nucleotide bases, or
(ii) if the nucleic acid of interest is single-stranded, obtaining a sample of the nucleic acid to serve as an unpaired-base template nucleic-acid sample comprising a template of unpaired nucleotide bases;

(b) obtaining an oligonucleotide primer having a nucleotide sequence capable of hybridising with the particular nucleotide sequence if the particular nucleotide sequence is present in the template nucleic acid to form a primer-extendable duplex between the primer and the particular nucleotide sequence such that the hybridised primer is extendable at a 3' end by a nucleic acid template-dependent, primer extension reaction;
(c) immobilising the oligonucleotide primer on a solid support, such as to permit the immobilised primer to hybridise with the template nucleic acid to form a primer-extendable duplex if the particular nucleotide sequence is present in the template nucleic acid such that the hybridised primer is extendable at the 3' end by a nucleic acid template-dependent, primer extension reaction;
(d) contacting the immobilised oligonucleotide primer under hybridising conditions with unpaired-base-template sample from step (a) to form on the solid support a primer-extendable duplex between the immobilised primer and the template nucleic acid if the particular nucleotide sequence is present in the template nucleic acid such that the hybridised primer is extendable at the 3' end by a nucleic acid template-dependent, primer extension reaction;
(e) contacting the primer-extendable duplex from step (d), if any, with a reagent composition which comprises an aqueous carrier and an admixture of four different terminators of a nucleic acid template-dependent, primer extension reaction, at least one of the terminators being labeled with a detectable marker, each of the terminators being capable of specifically terminating the primer extension reaction in a manner dependent on the identity of an unpaired nucleotide base in a template immediately adjacent to, and downstream of, the 3' end of a primer hybridised to the template such that the hybridised primer is extendable at a 3' end by the primer extension reaction, the primer-extendable duplex, if any, being contacted with the reagent composition under conditions permitting base pairing of a complementary terminator present in the reagent with a nucleotide base in the template nucleic acid of the duplex immediately adjacent to, and downstream of, the 3' end of the hybridised primer of the duplex and occurrence of the template-dependent, primer extension reaction so as to incorporate the complementary terminator at the 3' end of the primer to form an extended primer such that the primer is extended by the complementary terminator if the particular nucleotide sequence is present in the template nucleic acid, no such extended primer being formed if the particular nucleotide sequence is not present in the template nucleic acid;
(f) removing labeled terminator not incorporated in the primer from the solid support; and
(g) detecting the presence or absence of a detectable marker at the 3' end of the immobilised primer from step (e) on the solid support, to determine the presence or absence of the particular nucleotide sequence in the nucleic acid of interest.

[0038] In one embodiment, step (e) employs four labeled terminators, each terminator being labeled with a different detectable marker. The duplex of primer and the particular nucleotide sequence, if any, is contacted with the reagent under conditions permitting base pairing of a complementary terminator present in the reagent with the unpaired template nucleotide base downstream of the 3' end of the primer, the primer being hybridized with the particular nucleotide sequence in the template, and the occurrence of a template-dependent, primer extension reaction so as to incorporate the terminator at the 3' end of the primer. Next, the absence or presence and identity of a detectable marker at the 3'

end of the primer are determined. The presence or absence of the detectable marker indicates whether the primer has hybridized to the template. If a detectable marker is absent, the primer did not hybridize to the template, and, therefore, the particular nucleotide sequence is not present in the sample of nucleic acids. If a detectable marker is present, the primer did hybridize to the template, and, therefore, the particular nucleotide sequence is present in the sample of nucleic acids.

[0039] In another embodiment, only one of the terminators has a detectable marker. The duplex of primer and the particular nucleotide sequence, if any, is contacted with the reagent under conditions permitting base pairing of a complementary terminator present in the reagent with the unpaired template nucleotide base downstream of the 3' end of the primer, the primer being hybridized with the particular nucleotide sequence in the template, and the occurrence of a template-dependent, primer extension reaction. The net result is the incorporation of the terminator at the 3' end of the primer.

[0040] The original duplex of primer and the particular nucleotide sequence, if any, is then contacted with three different reagents, with a different one of each of the four terminators being labeled in each of the four parallel reaction steps. Next, the products of the four parallel, template-dependent, primer extension reactions are examined to determine which, if any, of the products have detectable markers. The absence or presence and identity of the detectable marker indicates whether the primer has hybridized to the template. If no detectable marker is present in any of the products, the primer did not hybridize to the template, and, therefore, the particular nucleotide sequence was not present in the sample of nucleic acids. If a detectable marker is present in any of the products, the primer did hybridize to the template, and, therefore, the particular nucleotide sequence was present in the sample of nucleic acids.

[0041] Different versions of the method for determining the identity of a nucleotide base at a specific position in a nucleic acid of interest and the method for determining the presence or absence of a particular nucleotide sequence in a sample of nucleic acids are possible. In the first version, the template is a deoxyribonucleic acid, the primer is an oligodeoxyribonucleotide, oligoribonucleotide, or a copolymer of deoxyribonucleotides and ribonucleotides, and the template-dependent enzyme is a DNA polymerase. This version gives a DNA product. In a second version, the template is a ribonucleic acid, the primer is an oligodeoxyribonucleotide, oligoribonucleotide, or a copolymer of deoxyribonucleotides and ribonucleotides, and the template-dependent enzyme is a reverse transcriptase. This version gives a DNA product. In a third version, the template is a deoxyribonucleic acid, the primer is an oligoribonucleotide, and the enzyme is an RNA polymerase. This version gives an RNA product. In a fourth version, the template is a ribonucleic acid, the primer is an oligoribonucleotide, and the template-dependent enzyme is an RNA replicase. This version gives an RNA product.

[0042] Preferably, before the primer extension reaction is performed, the template is capped by the addition of a terminator to the 3' end of the template. The terminator is capable of terminating a template-dependent, primer extension reaction. The template is capped so that no additional labeled terminator will attach at the 3' end of the template. The extension reaction should occur on the primer, not on the template. A dideoxynucleotide can be used as a terminator for capping the template.

[0043] Another modification of the method for determining the identity of a nucleotide base at a specific position in a nucleic acid of interest is to separate the primer from the nucleic acid of interest after the extension reaction by using appropriate denaturing conditions. The denaturing conditions can comprise heat, alkali, formamide, urea, glyoxal, enzymes, and combinations thereof. The denaturing conditions can also comprise treatment with 2.0 N NaOH.

[0044] The nucleic acid of interest can comprise non-natural nucleotide analogs such as deoxyinosine or 7-deaza-2'-deoxyguanosine. These analogues destabilize DNA duplexes and could allow a primer annealing and extension reaction to occur in a double-stranded sample without completely separating the strands.

[0045] The sample of nucleic acids can be from any source. The sample of nucleic acids can be natural or synthetic (i.e., synthesized enzymatically in vitro). The sample of nucleic acids can comprise deoxyribonucleic acids, ribonucleic acids, or copolymers of deoxyribonucleic acid and ribonucleic acid. The nucleic acid of interest can be a deoxyribonucleic acid, a ribonucleic acid, or a copolymer of deoxyribonucleic acid and ribonucleic acid. The nucleic acid of interest can be synthesized enzymatically in vivo, synthesized enzymatically in vitro, or synthesized non-enzymatically. The sample containing the nucleic acid or acids of interest can comprise genomic DNA from an organism, RNA transcripts thereof, or cDNA prepared from RNA transcripts thereof. The sample containing the nucleic acid or acids of interest can also comprise extragenomic DNA from an organism, RNA transcripts thereof, or cDNA prepared from RNA transcripts thereof. Also, the nucleic acid or acids of interest can be synthesized by the polymerase chain reaction.

[0046] The sample can be taken from any organism. Some examples of organisms to which the method of the subject invention is applicable include plants, microorganisms, viruses, birds, vertebrates, invertebrates, mammals, human beings, horses, dogs, cows, cats, pigs, or sheep.

[0047] The nucleic acid of interest can comprise one or more moieties that permit affinity separation of the nucleic acid of interest from the unincorporated reagent and/or the primer. The nucleic acid of interest can comprise biotin which permits affinity separation of the nucleic acid of interest from the unincorporated reagent and/or the primer via binding of the biotin to streptavidin which is attached to a solid support. The sequence of the nucleic acid of interest

can comprise a DNA sequence that permits affinity separation of the nucleic acid of interest from the unincorporated reagent and/or the primer via base pairing to a complementary sequence present in a nucleic acid attached to a solid support. The nucleic acid of interest can be labeled with a detectable marker; this detectable marker can be different from any detectable marker present in the reagent or attached to the primer.

[0048] The oligonucleotide primer can be an oligodeoxyribonucleotide, an oligoribonucleotide, or a copolymer of deoxyribonucleotides and ribonucleotides. The oligonucleotide primer can be either natural or synthetic. The oligonucleotide primer can be synthesized either enzymatically in vivo, enzymatically in vitro, or non-enzymatically in vitro. The oligonucleotide primer can be labeled with a detectable marker; this detectable marker can be different from any detectable marker present in the reagent or attached to the nucleic acid of interest. In addition, the oligonucleotide primer must be capable of hybridizing or annealing with nucleotides present in the nucleic acid of interest, immediately adjacent to, and upstream of, the nucleotide base to be identified. One way to accomplish the desired hybridization is to have the template-dependent primer be substantially complementary or fully complementary to the known base sequence immediately adjacent to the base to be identified.

[0049] The oligonucleotide primer can comprise one or more moieties that permit affinity separation of the primer from the unincorporated reagent and/or the nucleic acid of interest. The oligonucleotide primer can comprise biotin which permits affinity separation of the primer from the unincorporated reagent and/or nucleic acid of interest via binding of the biotin to streptavidin which is attached to a solid support. The sequence of the oligonucleotide primer can comprise a DNA sequence that permits affinity separation of the primer from the unincorporated reagent and/or the nucleic acid of interest via base pairing to a complementary sequence present in a nucleic acid attached to a solid support.

[0050] The subject invention also provides a method of typing a sample of nucleic acids which comprises identifying the base or bases present at each of one or more specific positions, each such nucleotide base being identified using one of the methods for determining the identity of a nucleotide base at a specific position in a nucleic acid of interest as outlined above. Each specific position in the nucleic acid of interest is determined using a different primer. The identity of each nucleotide base or bases at each position can be determined individually or the identities of the nucleotide bases at different positions can be determined simultaneously.

[0051] The subject invention also provides another method of typing a sample of nucleic acids which comprises determining the presence or absence of one or more particular nucleotide sequences, the presence or absence of each such nucleotide sequence being determined using one of the methods for determining the presence or absence of a particular nucleotide sequence in a sample of nucleic acids as outlined above.

[0052] The subject invention also provides an additional method of typing a sample containing nucleic acids. First, the presence or absence of one or more particular nucleotide sequences is determined; the presence or absence of each such nucleotide sequence is determined using one of the methods for determining the presence or absence of a particular nucleotide sequence in a sample of nucleic acids as outlined above. Second, the nucleotide base or bases present at each of one or more specific positions is identified; each such base is identified using one of the methods for determining the identity of a nucleotide base at a specific position in a nucleic acid of interest as outlined above.

[0053] The subject invention further provides a method for identifying different alleles in a sample containing nucleic acids which comprises identifying the base or bases present at each of one or more specific positions. The identity of each nucleotide base is determined by the method for determining the identity of a nucleotide base at a specific position in a nucleic acid of interest as outlined above.

[0054] The subject invention also provides a method for determining the genotype of an organism at one or more particular genetic loci which comprises obtaining from the organism a sample containing genomic DNA and identifying the nucleotide base or bases present at each of one or more specific positions in nucleic acids of interest. The identity of each such base is determined by using one of the methods for determining the identity of a nucleotide base at a specific position in a nucleic acid of interest as outlined above. The identity of the nucleotide bases determine the different alleles and, thereby, determine the genotype of the organism at one or more particular genetic loci.

[0055] The chain termination reagent in combination with an appropriate oligonucleotide primer, and a DNA polymerase with or without an associated 3' to 5' exonuclease function, and an appropriate salt and cofactor mixture, can be used under appropriate hybridization conditions as a kit for diagnosing or typing nucleic acids, if appropriate primer separation techniques are used. To simplify the primer separation and the terminal nucleotide addition analysis this invention makes use of oligonucleotides that are modified in such ways that permit affinity separation as well as polymerase extension. The 5' termini and internal nucleotides of synthetic oligonucleotides can be modified in a number of different ways to permit different affinity separation approaches, e.g., biotinylation. These affinity reagents can be used with the terminator mixture to facilitate the analysis of extended oligonucleotide(s) in two ways:

(1) If a single affinity group is used on the oligonucleotide(s), the oligonucleotide(s) can be separated from the unincorporated terminator reagent. This eliminates the need of physical or size separation.

(2) More than one oligonucleotide can be separated from the terminator reagent and analyzed simultaneously if

more than one affinity group is used. This permits the analysis of several nucleic acid species or more nucleic acid sequence information per extension reaction.

[0056] The affinity group(s) need not be on the priming oligonucleotide but could, alternatively, be present on the template. As long as the primer remains hydrogen bonded to the template during the affinity separation step, this will allow efficient separation of the primer from unincorporated terminator reagent. This also has the additional benefit of leaving sites free on the primer for the convenient attachment of additional moieties. For example, the 5'-terminus of the primer could be modified by coupling it to a suitable fluorescent group such as rhodamine, allowing the amount of primer in the primer:template complex to be easily quantified after the affinity separation step. The amounts of 3'-terminating terminators could then be normalized to the total amount of annealed primer.

[0057] The oligonucleotide primers and template can be any length or sequence, can be DNA or RNA, or any modification thereof. It is necessary, however, that conditions are chosen to optimize stringent hybridization of the primers to the target sequences of interest.

[0058] The conditions for the occurence of the template-dependent, primer extension reaction can be created, in part, by the presence of a suitable template-dependent enzyme. Some of the suitable template-dependent enzymes are DNA polymerases. The DNA polymerase can be of several types. The DNA polymerase must, however, be primer and template dependent. For example, E. coli DNA polymerase I or the "Klenow fragment" thereof, T4 DNA polymerase, T7 DNA polymerase ("Sequenase"), T. aquaticus DNA polymerase, or a retroviral reverse transcriptase can be used. RNA polymerases such as T3 or T7 RNA polymerase could also be used in some protocols. Depending upon the polymerase, different conditions must be used, and different temperatures ranges may be required for the hybridization and extension reactions.

[0059] The reagents of the subject invention permit the typing of nucleic acids of interest by facilitating the analysis of the 3' terminal addition of terminators to a specific primer or primers under specific hybridization and polymerase chain extension conditions. Using only the terminator mixture as the nucleoside triphosphate substrate ensures addition of only one nucleotide residue to the 3' terminus of the primer in the polymerase reaction. Using all four terminators simultaneously ensures fidelity, i.e., suppression of misreading.

[0060] By specifically labeling one or more of the terminators, the sequence of the extended primer can be deduced. In principle, more than one reaction product can be analyzed per reaction if more than one terminator is specifically labeled.

[0061] By specifically tagging the oligonucleotide primer(s), or template(s) with a moiety that does not affect the 3' extension reaction yet permits affinity separation, the extension product(s) can be separated post-reaction from the unincorporated terminators, other components of the reagents, and/or the template strand. Several oligonucleotides can be analyzed per extension reaction if more than one affinity agent is used.

[0062] In principle, the combination of four differently labeled terminators and many primers or templates tagged with different groups permits the typing of many different nucleic acid sequences simultaneously.

[0063] Specificity in this diagnostic reaction is determined by (1) the stringency of oligonucleotide hybridization and (2) the sequence information gained by the single residue extension.

A. General Methods

1. Biotinylation of oligodeoxynucleotides.

[0064] Oligodeoxynucleotides, terminated at their 5'-ends with a primary amino group, were ordered from Midland Certified Reagents, Midland, Texas. These were biotinylated using biotin-XX-NHS ester (Clontech Laboratories, Inc., Palo Alto, California), a derivative of biotin-N-hydroxysuccinimide. Reagents used were from the Clontech biotinylation kit. Typically, the oligonucleotide (9 nanomoles) was dissolved in 100 µl of 0.1M $NaHCO_3/Na_2CO_3$ (pH 9), and 25 µl of N,N-dimethylformamide containing 2.5 mg biotin-XX-NHS-ester was added. The mixture was incubated overnight at room. temperature. It was then passed over a 6. ml Sephadex (RTM) G-25 column ("DNA grade" - Pharmacia) equilibrated with H_2O. Eluate fractions containing DNA were identified by mixing 4 µl aliquots with an equal volume of ethidium bromide (2 µg/ml) and the DNA-induced fluorescence was monitored with a UV transilluminator. Unreacted ester was detected by UV absorption at 220nm. The tubes containing DNA were pooled, concentrated in a Centricon-3 (RTM) microconcentrator (Amicon), and passed over Sephadex again.

[0065] Inhibition of the binding of [^{3}H]-biotin to magnetic M-280 streptavidin Dynabeads (Dynal) was used to assay quantitatively the extent of biotinylation of the oligonucleotides. Eppendorf tubes and pipet tips were siliconized. A known amount (5-10 pmoles) of biotin-labeled oligonucleotide in 10 µl 0.1M NaCl was added to tubes containing 25 µl of 1:4 suspension of beads in 0.1M NaCl. The tubes were rotated for one hour on a Labquake shaker (Labindustries, Inc.). Increasing amounts of [^{3}H]-biotin (5-35 pmoles) in 20 µl of 0.1M NaCl were added to the tubes and these were rotated again for one hour. Tubes were put on a Dynal MPC-E magnet to remove the beads from suspension, 10 µl

aliquots of the supernatant were withdrawn, and the amount of radioactivity in these was measured using a Beckman LS 5000 TD liquid scintillation counter. counts were compared to those from tubes to which no oligonucleotide had been added. Alternatively, for some primers, biotinylation was monitored by size fractionation of the reaction products using analytical polyacrylamide gel electrophoresis in the presence of 8 M urea.

2. Template-dependent primer extension/termination reactions.

[0066] Approximately five pmoles of 5'-biotinylated oligodeoxynucleotide template (see above) were mixed with approximately three pmoles of primer in 1X sequencing buffer (from Sequenase Version 2.0 kit, US Biochemical Corp.) (10 µl final volume) , the mixture was incubated at 65°C for 2 min, then allowed to cool to room temperature in order to anneal the primer and template. The solution containing the annealed template-primer was separated into two 5 µl portions, A and B, to which were added the following: Reactions A (for normalizing template concentrations) - 0.5 µl of 100 mM dithiothreitol, 1 µl each of 10 µM dATP, dGTP, ddCTP, 0.5 µl of "Mn buffer" (from Sequenase Version 2.0 kit, US Biochemical Corp.), 0.5 µl of [^{35}S]-α-thio-dTTP (10 mCi/ml 1180 Ci/mmole) (Dupont-NEN), 1 µl of Sequenase (RTM) (1:8 dilution, US Biochemical Corp.); Reactions B (for template-specific labeling of primer 3'-ends) - same additions as in Reactions A except the nucleotides used were ddCTP, ddGTP, ddTTP, and [^{35}S]-α-thio-ddATP.

[0067] Reactions were for 5 min at 37°C. control reactions omitting the primer or the Sequenase were also performed. Aliquots were removed and analyzed by electrophoresis on a 15% polyacrylamide, 8 M urea, DNA sequencing gel (see Maniatis, T., et al., Molecular Cloning, a Laboratory Manual, cold Spring Harbor Laboratory (1982)). The gel was fixed in 10% methanol, 10% acetic acid, dried down onto Whatman's 3MM paper, and exposed to Kodak X-Omat AR film. Alternatively, for purposes of analyzing the products by liquid scintillation counting, the biotinylated template or template-primer was bound to an excess of M-280 streptavidin Dynabeads (Dynal) before or after the Sequenase reaction (see above, "1. Biotinylation of oligodeoxynucleotides", for binding conditions). Beads were washed three times with 0.1 M NaCl to remove unincorporated label, then scintillation fluid was added and the radioactivity measured by liquid scintillation counting.

3. Generation of templates from polymerase chain reaction products.

[0068] Polymerase chain reaction (PCR) reactions were carried out where one or the other of the amplification primers flanking the target stretch of DNA were biotinylated as described above. These primers (2 µmol final concentration) and the target DNA (up to 1 µg) were incubated with 2.5 units of Taq polymerase (Perkin Elmer/Cetus), 200 µM each of dATP, dCTP, dGTP, and dTTP, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, and 0.01% gelatin (Sigma). Reaction mixtures were-overlayed with paraffin oil and incubated for 30 cycles in Perkin Elmer/Cetus thermocycler. Each cycle consisted of 1 min at 94°C, 2 min at 60°C, and 3 min at 72°C. Reaction products were purified by phenol/chloroform extraction and ethanol precipitation, then analyzed by ethidium bromide staining after electrophoresis on a polyacrylamide gel. The yield of duplex PCR product was typically about 10 µg.

[0069] Approximately 5 µg of this PCR product was incubated with gentle agitation for 60 min with 50 µL of a suspension of prewashed M-280 Dynabeads in 0.1 M NaCl. The beads with the bound DNA (approximately 15 pmoles) were then incubated for 5 min at 25°C with 0.15 M NaOH. Beads were washed once with 0.15 M NaOH to remove the unbiotinylated DNA strand, then washed three times with H_2O. The beads were resuspended in H_2O and the strand bound to the beads via the biotin-streptavidin link was used as template for further primer extension reactions.

B. Examples

Example 1

[0070]

Primer oligo 182: $^{5'}$ GCCTTGGCGTTGTAGAA $^{3'}$

Template oligos
180(C)/181(T): $^{3'}$ TCGGGTCGGAACCGCAACATCTT^{C}/TATAGACTA $^{5'}$

[0071] Oligonucleotides 180 and 181 were synthesized with primary amino groups attached to their 5' termini. These

were coupled with biotin as described above. Oligonucleotide 182 was annealed as a primer and extension reactions "A" and "B" (see above) were carried out. The expected template-dependent 3'-terminal extensions to oligonucleotide 182 were as follows ("*" preceding a nucleotide signifies a radioactive label):

Template	Reaction A	Reaction B
180	-dG-*dT-dA-*dT-ddC	-ddG
181	-dA-*dT-dA-*dT-ddC	-*ddA

[0072] Thus, in the "A" reactions, both template oligonucleotides will direct a radioactively-labelled five nucleotide extension of the primer; the amount of labeling should be proportional to the amount of productively primed template present in the reactions. In the "B" reactions, both templates will direct a one nucleotide extension of the primer, but only for template 181 should this result in labeling of the primer. The "B" reaction, therefore, is an example of template-directed, sequence-specific labeling of an oligonucleotide via DNA polymerase-catalyzed extension of a productive primer-template complex.

[0073] The reaction products were fractionated by size on a 15% polyacrylamide/8M urea sequencing gel and visualized by autoradiography. The results (Figure 1) show that, as expected, the "A" reactions yield labeling and extension of both primers whereas the "B" reaction results in labeling that is strongly biased in favor of template 181. Panel C in Figure 1 shows a gel analysis of the same reaction products as in Panel B, except the reaction products were first purified as described above using M-280 streptavidin Dynabeads.

Example 2

[0074] The experiment described in Example 1 shows template-directed labeling of oligonucleotide primer 182 in which the labeling is specific with respect to oligonucleotides or other species that migrate similarly on a polyacrylamide gel. In order to assess more generally the template-directed specific labeling of oligonucleotide 182 with respect to all other labeled species, regardless of gel mobility, a direct measurement of incorporated radioactivity was performed. In this experiment, both reactions "A" and "B" were performed, reaction products were purified using Dynabeads, and total radioactivity in the aliquots was measured by liquid scintillation counting. This procedure assesses both misincorporation of label into other species and, in addition, the efficiency of the Dynabead washing procedure with respect to unincorporated nucleotides. As a practical matter, it would be of interest to minimize both sources of non-specific label in order to have a simple, non-gel-based, procedure for assessing specific, template-directed labeling of the primer. The results of directly counting the reaction products after washing on the magnetic beads are as follows (all results expressed as cpm of ^{35}S):

Reaction	Template 180	Template 181
A, complete	325,782	441,823
A, no polymerase	5,187	5,416
A, no primer	4,351	12,386
B, complete	5,674	176,291
B, no polymerase	2,988	1,419
B, no primer	1,889	1,266

[0075] As can be seen from these results, specific template-directed labeling of primer 182 can also be determined by measuring the total radioactivity of the reaction products after washing with magnetic beads to remove unreacted nucleotides. The background in this experiment due to nonspecific label from all other sources was approximately 3-4% (compare templates 180 and 181 in the "B, complete" reaction). Control experiments ("no polymerase" and "no primer") showed that the bulk of the background label was probably contributed by unincorporated nucleotides that were not completely removed by the washing step. The "A, complete" reactions showed that, for both templates, productive template:primer complexes were present.

Example 3

[0076] Two amplification primers, TGL 105 and TGL 106 (Figure 2), were used to amplify a cloned stretch of bovine DNA containing two DNA sequence polymorphisms: a C or T at position 114 and an A or G at position 190 (Figure 2).

DNAs containing these polymorphisms were molecularly cloned and available on plasmids, as follows: plasmid p183, C114 and A190; plasmid p624, T114 and A190; plasmid p814, C114 and G190. Four PCR reactions with biotinylated primers were performed to amplify and purify specific strands of these plasmids for use as templates:

Primers	Plasmids	Detection Primers
105 biotinylated, 106 unbiotinylated	p183 and p624	TGL 182
105 unbiotinylated, 106 biotinylated	p183 and p814	TGL 166

[0077] The duplex PCR products were bound to magnetic microspheres, denatured with NaOH, and the biotinylated strand purified as described above. Templates prepared with biotinylated TGL 105 were subjected to analysis by DNA sequencing with unbiotinylated primer TGL 106 in order to measure the amount of template present. Similarly, template prepared using biotinylated TGL 106 was analyzed by sequencing with unbiotinylated TGL 105.

[0078] Approximately equal amounts of template (2 pmoles) were annealed for 5 min at 65°C to the polymorphism detection primers, TGL 182 and TGL 166 (see above and Figure 2). These primers hydrogen-bond to the templates in a sequence-specific fashion such that their 3'-termini are adjacent to nucleotide positions 114 and 190, respectively (Figure 2). Template-directed primer extension reactions (reaction "B" conditions) were carried out on these primer: template complexes in the presence of the four ddNTPs, one of which (ddATP) was labeled. The products of these extension reactions were analyzed by electrophoresis on a 15% polyacrylamide/8M urea gel followed by autoradiography (Figure 3).

Example 4

[0079]

Primer oligo TGL391: $^{5'}$TGTTTTGCACAAAAGCA$^{3'}$

Primer oligo TGL346: $^{5'}$GTTTTGCACAAAAGCAT$^{3'}$

Template oligo TGL382: $^{3'}$CACAAAACGTGTTTTCGTAGGA$^{5'}$-biotin:(streptavidin-bead)

[0080] Oligonucleotide TGL382 was purchased from the Midland Certified Reagent Company, Midland, Texas. It was biotinylated using Midland Certified Reagent Company's "Biotin dX" reagent (a biotin derivative phosphoramidite) which is suitable for use in automated DNA synthesis in the 5' terminal nucleotide position. The biotinylated oligonucleotide was then purified by anion exchange HPLC. Streptavidin-conjugated M-280 Dynabeads were washed in TNET buffer (10mM Tris-HCl, pH 7.5, 100mM NaCl, 1mM EDTA, 0.1% Triton X-100) and resuspended in the same buffer at a concentration of 7 X 10^8 beads/ml. 10-100 pmoles of biotinylated oligonucleotide TGL382 was incubated with 100 µl of the Dynabead suspension in TNET for 30 minutes at 20°C in order to allow the biotin moiety to bind to the streptavidin. The beads were then washed (using a magnet to immobilize them) three times with 200 µl of TNET and resuspended in 100 µl of TNET. For annealing, 25 µl of this suspension of the Dynabeads with the attached template oligonucleotide was immobilized with the magnet, the TNET withdrawn, and 25 µl of 40 mM Tris-HCL, pH 7.5, 20 mM $MgCl_2$, 50 mM NaCl, containing 2 µM of oligonucleotide primers 346 or 391, was added. The template and each primer were annealled by incubating them for 5 minutes at 65°C, followed by slow cooling over a period of 20 minutes to room temperature. Beads containing the bound template-primer complexes were washed twice with 200 µl TNET, followed by resuspension in 25 µl of 40 mM Tris-HCl, pH 7.5, 20 mM $MgCl_2$, 50 mM NaCl.

[0081] The following ddNTP mixes were used:

[0082] ^{35}S-labelled dideoxynucleoside triphosphate mixes (labelled nucleotide indicated in the form ddN*TP):

ddG Mix	5 μM ddG*TP	10 μM ddATP	10 μM ddTTP
	10 μM ddCTP		
ddA Mix	10 μM ddGTP	5 μM ddA*TP	10 μM ddTTP
	10 μM ddCTP		
ddT Mix	10 μM ddGTP	10 μM ddATP	5 μM ddT*TP
	10 μM ddCTP		
ddC Mix	10 μM ddGTP	10 μM ddATP	10 μM ddTTP
	5 μM ddC*TP		

[0083] The ddN*TPs were the four respective [α-thio-^{35}S]dideoxynucleoside triphosphates (purchased from New England Nuclear).

[0084] For each bead-bound, template-primer complex, four extension reactions were carried out, one reaction for each of the four ddNTP mixes. Extension reactions contained the following components: 5.0 μl bead suspension containing the annealled template-primer complex, 0.5 μl of 100 mM dithiothreitol, 0.5 μl of "Mn^{++} solution" (100 mM $MnCl_2$, 150 mM DL-isocitrate, pH 7.0; purchased from U.S. Biochemicals, Cleveland, Ohio), 1.0 μl of ddG, ddA, ddT, or ddC mix, 2.0 μl of H_2O, and 1.0 μl of T7 DNA polymerase ("Sequenase", version 2.0, US Biochemicals, 1625 units/ml in 50 mM Tris-HCl, pH 7.5, 10 mM 2-mercaptoethanol, 1 mg/ml bovine serum albumin).

[0085] Reactions were allowed to proceed for 15 minutes at 20°C, then stopped by washing the magnetically immobilized beads three times with 500 μl TNET. Beads were resuspended in final volume of 25 μl TNET prior to the detection assays.

[0086] Incorporation of labelled dideoxynucleotides by the primer extension reaction was assayed two different ways: gel electrophoresis followed by autoradiography, and direct autoradiographic analysis of labelled DNA.

1. Gel electrophoresis followed by autoradiography (^{35}S-labelled material only). Samples of washed, bead-bound DNA were heated at 94°C for 5 minutes in 10 μl of formamide loading buffer (80% formamide, 10 mM Tris-HCl, pH 8, 1 mM EDTA, 0.02% bromphenol blue) to denature the DNA and release the labelled primer from the primer: template complex. Samples were analyzed by electrophoresis on 8 or 12.5% polyacrylamide/8 M urea sequencing gels (19:1 acrylamide:bis-acrylamide ratio; 100 mM Tris-HCl, 100 mM borate, 2 mM EDTA, pH 8.3, running buffer; 60 watts constant power). After electrophoresis, gels were either dried down onto filter paper or frozen at -80°C to prevent diffusion, covered with plastic wrap, and exposed to X-ray film to visualize the labelled DNA by autoradiography (Figure 4).

2. Direct autoradiographic analysis of labelled DNA. For the analysis of total radioactivity bound to the beads, 10 μl aliquots of the bead suspensions in TNET were spotted directly onto filter paper or nylon membranes. Filters or membranes were dried under an incandescent lamp, covered with plastic wrap, and exposed to X-ray film (Figure 5).

Example 5

[0087]

TGL240: $^{5'}$AGATGATGCTTTTGTGCAAAACAC$^{3'}$

TGL239: $^{5'}$TCAATACCTGAGTCCCGACACCCTG$^{3'}$

TGL308: $^{5'}$AGCCTCAGACCGCGTGGTGCCTGGT$^{3'}$

[0088] Oligonucleotide TGL240 was synthesized with a primary amino group attached to its 5' terminus and coupled

with biotin as described above. TGL240 (biotinylated) and TGL239 (unbiotinylated) were used to amplify, via the polymerase chain reaction procedure (see "A. General Methods"), a region of DMA comprising a particular genetic locus in samples of mammalian genomic DNA. DNAs from two different individuals, each homozygous for a particular set of linked sequence polymorphisms (the "A" allele and the "B" allele -- see Figure 6), were examined. After the PCR reaction, 2-20 pmoles of duplex PCR DNA was incubated with 100 µl of streptavidin-conjugated M-280 Dynabeads (7 X 10^8 beads/ml) in TNET buffer in order to bind the biotinylated strand to the beads. After binding, the beads were magnetically immobilized and washed three times with 200 µl of TNET, then resuspended in 100 µl of TNET. To remove the non-biotinylated strand, 500 µl of 0.15 N NaOH was added and the suspension incubated for 30 minutes at 20°C. The beads were then magnetically immobilized and washed once with 250 µl of 0.15 N NaOH, three times with 500 µl TNET, and resuspended in 100 µl of TNET.

[0089] The detection primer, oligonucleotide TGL308 (Figure 6), was annealled to the bead-bound PCR-generated template as described above in Example 4. Further washes, extension reactions, and detection assays were also carried out as described in Example 4. A gel autoradiographic analysis of the labelled primer extension products for the two homozygous individuals, ESB164 ("AA" genotype) and EA2014 ("BB" genotype), is shown in Figure 7. Autoradiographic analyses of total bead-bound radioactivity, or primer-associated radioactivity after NaOH elution, are shown for these same individuals using the filter spotting assay (Figure 8). For the analysis of primer only, 10 µl of 0.4 N NaoH was added to 10 µl of the bead suspension. After incubation for 10 minutes at room temperature, the beads were immobilized magnetically and the supernatant withdrawn and spotted onto nylon blotting membrane.

Example 6

[0090] DNA Samples. Genomic DNA was isolated using the SDS/Proteinase K procedure (Maniatis, T. Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, 1989) from peripheral blood nucleated cells of humans or horses enriched from red blood cells by selective lysis accomplished by diluting blood with a three fold volume excess of ACK lysing buffer (0.15 M ammonium chloride, 1 mM potassium bicarbonate, 0.1 mM EDTA). Oligonucleotides were prepared by solid-phase phosphoramidite chemistry using an Applied Biosystems, Inc. (Foster City, CA) Model 391 automated DNA synthesizer. In the case of primers used in Genetic Bit Analysis (GBA) reactions, detritylation was not performed following the final cycle of synthesis and the full-length oligonucleotide was purified using the Applied Biosystems oligonucleotide purification cartridge (OPC) as recommended by the manufacturer. For most PCR reactions, primers were used directly by drying down the de-protection reaction. Oligonucleotides derivatized with 5'-amino groups were prepared using Aminolink 2 purchased from Applied Biosystems and used according the manufacturer's recommendations.

[0091] Oligonucleotide Sequences. Primers for first round amplification of equine locus JH85 were #91:

5' CGTCTGCAGAATCCACTGGCTTCTTGAG 3'

and #92:

5' GCAGGATCCTGGAACTACTCATTTGCCT 3'.

Second round amplification of equine locus was achieved using nested primers #239:

5' TCAATACCTGAGTCCCGACACCCTG 3'

and #240:

5' AGGATGATGCTTTTGTGCAAAACAC 3'

Amplification of human HLA DPA1 sequences (Marsh, S.G.E., Bodmer, J.G. HLA Class II Nucleotide Sequences, 1991. Human Immunol. 31:207-227) was accomplished with primers #467:

5' GCGGACCATGTGTCAACTTAT 3'

and #445:

5' GCCTGAGTGTGGTTGGAACTG 3'.

[0092] Template Preparation. Amplification of genomic sequences was performed using the polymerase chain reaction (PCR) (Saiki, R.K., Gelfand, D.H., Stoffel, S., Scharf, S.J., Higuchi, R., Horn, G.T., Mullis, K.B., Erlich, H.A., Primer Directed Enzymatic Amplification of DNA with a Thermostable DNA Polymerase. Science 239:487-491). In a first step, one hundred nanograms of genomic DNA was used in a reaction mixture containing each first round primer at a concentration of 2 μM/10 mM Tris pH 8.3/50 mM KCl/1.5 mM $MgCl_2$/0.1% gelatin/0.05 units per μl Taq DNA Polymerase (AmpliTaq, Perkin Elmer Cetus, Norwalk, CT). Reactions were assembled and incubated at 94°C for 1.5 minutes, followed by 30 cycles of 94°C/1 minute, 60°C/2 minutes, 72°C/3 minutes. Single stranded DNA was prepared in a second "asymmetric" PCR in which the products of the first reaction were diluted 1/1000. One of the primers was used at the standard concentration of 2 μM while the other was used at 0.08 μM. Under these conditions, both single stranded and double stranded molecules were synthesized during the reaction.

[0093] Solid phase immobilization of nucleic acids. Reactions were performed in 96-well plates (Nunc Nunclon plates, Roskilde, Denmark). The GBA primer was covalently coupled to the plate by incubating 10 pmoles of primer having a 5' amino group per well in 50 μl of 3 mM sodium phosphate buffer, pH 6, 20 mM 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) overnight at room temperature. After coupling, the plate was washed three times with 10mM Tris pH 7.5/150mM NaCl/.05% Tween-20 (TNTw).

[0094] Biotinylated ddNTPs. Biotinylated ddNTPs were synthesized according to U.S. Patent No. 5,047,519.

[0095] Hybridization, extension and detection in Microwell Plates. Hybridization of single-stranded DNA to primers covalently coupled to 96-well plates was accomplished by adding an equal volume of 3M NaCl/50 mM EDTA to the second round asymmetric PCR and incubating each well with 20 μl of this mixture at 55°C for 30 minutes. The plate was subsequently washed three times with TNTw. Twenty (20) μl of polymerase extension mix containing ddNTPs (3 μM each, one of which was biotinylated/5 mM DTT/7.5 mM sodium isocitrate/5 mM $MnCl_2$/0.04 units per μl of modified T7 DNA polymerase and incubated for 5 minutes at room temperature. Following the extension reaction, the plate was washed once with TNTw. Template strands were removed by incubating wells with 50 μl 0.2N NaOH for 5 minutes at room temperature, then washing the wells with another 50 μl 0.2N NaOH. The plate was then washed three times with TNTw. Incorporation of biotinylated ddNTPs was measured by an enzyme-linked assay. Each well was incubated with 20 μl of streptavidin-conjugated horseradish peroxidase (1/1000 dilution in TNTw of product purchased from BRL, Gaithersburg, MD) with agitation for 30 minutes at room temperature. After washing 5 times with TNTw, 100 μl of o-phenylenediamine (OPD, 1 mg/ml in 0.1 K Citric acid, pH 4.5) (BRL) containing 0.012% H_2O_2 was added to each well. The amount of bound enzyme was determined by photographing the plate after stopping the reaction or quantitatively using a Molecular Devices model "Vmax" 96-well spectrophotometer.

[0096] In order to demonstrate the generality of the procedure, the ability to type three different sites located on two different template molecules is shown. In the middle of figures 9 through 11 is shown the polymorphic region of these loci together with the sequence of the primers used to genotype the DNA samples. The genotype of the test DNA samples was previously determined by restriction analysis and gel electrophoresis (equine samples) or by allele specific hybridization (human samples).

[0097] At the top and bottom of Figures 9 through 11 are photographs of the non-radioactive analysis of these sites. Analysis of the "plus" strand (which corresponds to the mRNA for the HLA DPA1 but is arbitrarily chosen for the equine locus JH85) is shown at the top of the figure, analysis of the "minus" strand is shown in the lower photograph. Using horseradish peroxidase activity genotyping data was observed visually. Because both strands were suitable templates, it was possible to get genotypic confirmation by using two different primers. For the HLA DPA1 locus, two sites of variation were typed (Figures 9 and 10). Identical results were achieved. Spectrophotometric quantitation of a separate experiment involving the equine locus JH85 is shown in Figure 12. The average ratio of signals obtained with expected vs. inappropriate base incorporation was 62.2.

C. EMBODIMENTS

[0098] An example of one method to practice the present invention involves obtaining from a convenient source, such as blood, epithelium, hair, or other tissue, samples of DNA or RNA, then amplifying in vitro specific regions of the nucleic acid using the polymerase chain reaction, transcription-based amplification (see Kwoh, et al., Proc. Natl. Acad. Sci. 80:1173 (1989)), etc. Amplification is accomplished using specific primers flanking the region of interest, with one or more of the primers being modified by having an attached affinity group (although in any given reaction only one such primer is modified at a time). A preferred modification is attachment of biotin moieties to the 5'-termini of the primers. A sample (typically, 0.5 - 5 pmoles) of the amplified DNA is then bound to streptavidin-conjugated magnetic

microspheres (e.g., Dynal M-280 "Dynabeads") via the attached biotin moiety on the amplification primer. The DNA is denatured by adjusting the aqueous suspension containing the microspheres to a sufficiently alkaline pH, and the strand bound to the microspheres via the biotin-streptavidin link is separated from the complementary strand by washing under similar alkaline conditions. To accomplish this, the microspheres are centrifuged or immobilized by the application of a magnetic field. The microsphere-bound strand is then used as a template in the remaining manipulations.

[0099] To the template strand, generated as described above, a specific primer oligonucleotide is bound under high stringency annealing conditions, the sequence of the primer being consistent with unique binding to a site on the template strand immediately adjacent to a known DNA sequence polymorphism. A preferred sequence and mode of binding for the primer ensures that the primer forms a duplex with the template such that the 3'-terminal nucleotide of the primer forms a Watson-Crick basepair with the template nucleotide immediately adjacent to the site of the first nucleotide in the sequence polymorphism, without the duplex overlapping any of the polymorphic sequence to be analyzed. This arrangement causes the nucleotides added via template-directed, DNA polymerase-catalyzed, extension of the primer to be determined unambiguously by the polymorphic nucleotide sequence in the template.

[0100] The above-described primer: template complex is contacted, under conditions of salt, pH, and temperature compatible with template-directed DNA synthesis, with a suitable DNA polymerase and four different chain-terminating nucleotide analogues known to form specific base pairs with the bases in the template. Most likely, but not necessarily, the bases in the template as well as the chain-terminating analogues are based on the common nucleosides: adenosine, cytosine, guanine or inosine, thymidine or uridine. A preferred set of chain-terminating analogues are the four dideoxynucleoside triphosphates, ddATP, ddCTP, ddGTP, and ddTTP, where each of the four ddNTPs has been modified by attachment of a different fluorescent reporter group. These fluorescent tags would have the property of having spectroscopically distinguishable emission spectra, and in no case would the dideoxynucleoside triphosphate modification render the chain-terminating analogue unsuitable for DNA polymerase-catalyzed incorporation onto primer 3'-termini. The result of DNA polymerase-catalyzed chain extension in such a mixture with such a primer:template complex is the quantitative, specific and unambiguous incorporation of a fluorescent chain-terminating analogue onto the 3'-terminus of the primer, the particular fluorescent nucleotide added being solely dictated by the sequence of the polymorphic nucleotides in the template.

[0101] The fluorescently-tagged primer:template complex, still attached to the magnetic microspheres, is then separated from the reaction mix containing the unincorporated nucleotides by, for example, washing the magnetically immobilized beads in a suitable buffer. Additionally, it is desirable in some circumstances to then elute the primer from the immobilized template strand with NaOH, transfer the eluted primer to a separate medium or container, and subsequently determine the identity of the incorporated terminator. The identity of the attached fluorescent group is then assessed by illuminating the modified DNA strand with light, preferably provided by a laser, of a suitable wavelength and intensity and spectrophotometrically analyzing the emission spectrum produced. In general, for a two allels (diploid) system at any given site in the DNA sequence, there are ten possible canonical emission spectra produced, corresponding to the sixteen possible homozygotic and heterozygotic pairings. By suitable matching of the measured spectra to this library of canonical spectra it is possible to identify which chain-terminating nucleotide(s) have been added to the 3'-terminus of the primer and thereby identify the nature of the sequence polymorphism in the template. Spectra produced by multiple allele systems or by alleles present in a ratio other than 1:1 can also be deconvolved by suitable mathematical treatments to identify and estimate the relative ratios of each contributing nucleotide.

[0102] All of the above steps involve chemistries, manipulations, and protocols that have been, or are amenable to being, automated. Thereby, incorporation of the preferred mode of practice of this invention into the operation of a suitably programmed robotic workstation should result in significant cost savings and increases in productivity for virtually any diagnostic procedure that depends on the detection of specific nucleotide sequences or sequence differences in nucleic acids derived from biological samples.

[0103] Several features of the above-described method have been improved and constitute a preferred embodiment of subject invention. Specifically, it is possible to present a more convenient solid phase. Magnetic microspheres must be manipulated with care in order to effectively wash and resuspend them. It is therefore difficult to envisage high volume, automated assays using these beads. Furthermore, they are deeply colored and are not adapted to calorimetric or fluorescent assays.

[0104] The methodology of the invention has been adapted to allow the utilization of standard, polystyrene, 96-well microplates. These have the advantage of being widely used in clinical and research laboratories. There are a large number of liquid handling systems, including automated systems, adapted to this format. They are suited to optical signal detection methods and automated plate readers for different types of light detection are available.

[0105] The template will always come from the nucleic acid sample of interest. These nucleic acids may be from a sample suspected of containing an infectious agent, one from an individual whose genotype is being determined, a sample from a patient suspected of having cancer, etc. If the immobilized partner of the hybrid complex to be extended is the template, each nucleic acid sample would have to be treated in such a way as to make immobilization possible. On the other hand, the primer for a given nucleic acid position to be interrogated will always be the same. Therefore,

methods have been devised which allow the binding of the primer to the microplates and hybridization of single stranded template molecules to the plate-bound primer. This provides the additional feature of being able to make use of single-stranded templates produced in many different ways, including direct analysis of RNA.

[0106] Radioactive methods are inconvenient and produce waste which is difficult to dispose of. For this reason, most commercial biochemistry detection systems have been converted to non-radioactive methods. By using ddNTPs which are labeled with biotin, the method of the invention can be performed non-radioactively using a variety of detection systems including enzyme linked colorimetric assays.

[0107] Quality control is an important issue for tests designed to be used in clinical settings. Because the method of the invention interrogates the nucleic acid sequence itself, on double stranded molecules, there is an opportunity to derive complementary genetic information by independently interrogating both strands. Applicants have shown that this approach is feasible using both equine and human genetic variants.

[0108] In the previously described method, the template was prepared by PCR using derivatized primers to permit immobilization of the template on the solid phase. Derivitization of the template is no longer necessary when the primer is immobilized. Rather, using unequal concentrations of PCR primers in an otherwise standard PCR, it is possible to generate an excess of one single-stranded molecule or the other, depending on which primer is in excess. These serve as convenient templates for hybridization to plate-bound primer molecules.

Claims

1. A method of determining the identity of a nucleotide base at a specific position in a nucleic acid of interest which comprises:

   (a) in the alternative:

   (i) if the nucleic acid of interest is double-stranded, treating a sample containing the nucleic acid so as to obtain an unpaired-base template nucleic-acid sample comprising a template of unpaired nucleotide bases spanning the specific position, or
   (ii) if the nucleic acid of interest is single-stranded, obtaining a sample of the nucleic acid to serve as an unpaired-base template nucleic-acid sample comprising a template of unpaired nucleotide bases spanning the specific position;

   (b) obtaining an oligonucleotide primer having a nucleotide sequence capable of hybridising with the template nucleic acid to form a duplex wherein the nucleotide base to be identified is immediately adjacent to, and downstream of, the 3' end of the primer;
   (c) immobilising the oligonucleotide primer on a solid support, such as to permit the immobilised primer to hybridise with the template nucleic acid to form a duplex wherein the nucleotide base to be identified is immediately adjacent to, and downstream of, the 3' end of the primer;
   (d) contacting the immobilised oligonucleotide primer under hybridising conditions with unpaired-base-template sample from step (a) to form on the solid support a duplex between the immobilised primer and the template nucleic acid wherein the nucleotide base to be identified is immediately adjacent to, and downstream of, the 3' end of the primer in the duplex;
   (e) contacting the duplex from step (d) with a reagent composition which comprises an aqueous carrier and an admixture of four different terminators of a nucleic acid template-dependent, primer extension reaction, each of the terminators being capable of specifically terminating the extension reaction in a manner strictly dependent on the identity of the unpaired nucleotide base in the template immediately adjacent to, and downstream of, the 3' end of a primer, wherein one of the terminators is complementary to the nucleotide base to be identified and at least one of the terminators is labeled with a , detectable marker, under conditions permitting base pairing of the complementary terminator present in the reagent with the nucleotide base to be identified and occurrence of a template-dependent, primer extension reaction so as to incorporate the complementary terminator at the 3' end of the primer to form an extended primer such that the primer has been extended by the complementary terminator;
   (f) removing labeled terminator not incorporated in the primer from the solid support; and
   (g) identifying any detectable marker present at the 3' end of the immobilised extended primer from step (e) on the solid support, to determine the identity of the nucleotide base to be identified.

2. A method according to claim 1 wherein each of the four terminators is labeled with a detectably different detectable marker.

3. A method as claimed in claim 1 wherein the oligonucleotide primer is immobilised at one of a plurality of separate test locations on the solid support.

4. A method as claimed in claim 3 wherein steps (c) to (g) are repeated four times with the same oligonucleotide primer from step (b) and the same unpaired-base-template sample from step (a) at four separate test locations on the solid support, with a corresponding one of four different reagent compositions being used in step (e) at each of the four test locations, in each of the four reagent compositions only one of the terminators being labeled with a detectable marker, a different one of the four terminators being labeled in each of the four different reagent compositions, so that identifying a detectable marker present at the 3' end of the immobilised extended primer from step (e) at one of the four test locations in step (g) determines the identity of the nucleotide base to be identified.

5. A method as claimed in any one of the preceding claims, wherein in the reagent of step (e), the terminators comprise a nucleotide or nucleotide analog.

6. A method according to claim 5 wherein the terminators comprise a dideoxynucleotide or an arabinoside triphosphate.

7. A method according to claim 6 wherein the terminators comprise one or more of ddATP, ddCTP, ddGTP or ddTTP.

8. A method of typing a sample containing nucleic acids which comprises identifying the nucleotide base or bases present at each of one or more specific positions, each such nucleotide base being identified using a method as claimed in any one of claims 1 to 7 and each such specific position being determined using a different primer and wherein the identity of each nucleotide base or bases at each position is determined individually or wherein the identities of the nucleotide bases at different positions are determined simultaneously.

9. A method for identifying different alleles in a sample containing nucleic acids which comprises identifying the nucleotide base or bases present at each of one or more specific positions, each such nucleotide base being identified by a method as claimed in any one of claims 1 to 7.

10. A method for determining the genotype of an organism at one or more particular genetic loci which comprises:

    (a) obtaining from the organism a sample containing genomic DNA; and
    (b) identifying the nucleotide base or bases present at each of one or more specific positions in nucleic acids of interest, each such base or bases being identified using a method as claimed in any one of claims 1 to 7 and thereby identifying different alleles and thereby, in turn, determining the genotype of the organism at one or more particular genetic loci.

11. A method as claimed in any one of claims 1 to 7 wherein, prior to the primer extension reaction in step (e), the template has been capped at its 3' end by the addition of a terminator to the 3' end of the template, said terminator being capable of terminating a template-dependent, primer extension reaction.

12. A method as claimed in any one of claims 1 to 7, wherein the sample comprises genomic DNA from an organism, including a plant, microorganism, virus, bird, vertebrate or invertebrate, mammal, human being, horse, dog, cow, cat, pig or sheep.

13. A method as claimed in any one of claims 1 to 7, wherein the primer is complementary to the known base sequence immediately adjacent to the base to be identified.

14. A method as claimed in any one of claims 1 to 7, wherein the primer is separated from the nucleic acid of interest after the primer extension reaction in step (c) by using appropriate denaturing conditions, including heat, alkali, formamide, urea, glyoxal, enzymes or combinations thereof.

15. A method as claimed in claim 1 wherein the terminators labeled with detectable markers are labeled with detectable markers which are nonradioactive.

16. A method as claimed in claim 15 wherein the step (g) of identifying any detectable marker at the 3' end of the immobilised extended primer is carried out by visual or optical means.

17. A method as claimed in claim 16 wherein the solid support is adapted for an optical signal detection method.

18. A method as claimed in claim 17 wherein the solid support is adapted for optical signal detection using an automated plate reader.

19. A method of determining the presence or absence of a particular nucleotide sequence in a nucleic acid of interest which comprises:

(a) in the alternative:

(i) if the nucleic acid of interest is double-stranded, treating a sample containing the nucleic acid so as to obtain an unpaired-base template nucleic-acid sample comprising a template of unpaired nucleotide bases, or
(ii) if the nucleic acid of interest is single-stranded, obtaining a sample of the nucleic acid to serve as an unpaired-base template nucleic-acid sample comprising a template of unpaired nucleotide bases;

(b) obtaining an oligonucleotide primer having a nucleotide sequence capable of hybridising with the particular nucleotide sequence if the particular nucleotide sequence is present in the template nucleic acid to form a primer-extendable duplex between the primer and the particular nucleotide sequence such that the hybridised primer is extendable at a 3' end by a nucleic acid template-dependent, primer extension reaction;
(c) immobilising the oligonucleotide primer on a solid support, such as to permit the immobilised primer to hybridise with the template nucleic acid to form a primer-extendable duplex if the particular nucleotide sequence is present in the template nucleic acid such that the hybridised primer is extendable at the 3' end by a nucleic acid template-dependent, primer extension reaction;
(d) contacting the immobilised oligonucleotide primer under hybridising conditions with unpaired-base-template sample from step (a) to form on the solid support a primer-extendable duplex between the immobilised primer and the template nucleic acid if the particular nucleotide sequence is present in the template nucleic acid such that the hybridised primer is extendable at the 3' end by a nucleic acid template-dependent, primer extension reaction;
(e) contacting the primer-extendable duplex from step (d), if any, with a reagent composition which comprises an aqueous carrier and an admixture of four different terminators of a nucleic acid template-dependent, primer extension reaction, at least one of the terminators being labeled with a detectable marker, each of the terminators being capable of specifically terminating the primer extension reaction in a manner dependent on the identity of an unpaired nucleotide base in a template immediately adjacent to, and downstream of, the 3' end of a primer hybridised to the template such that the hybridised primer is extendable at a 3' end by the primer extension reaction, the primer-extendable duplex, if any, being contacted with the reagent composition under conditions permitting base pairing of a complementary terminator present in the reagent with a nucleotide base in the template nucleic acid of the duplex immediately adjacent to, and downstream of, the 3' end of the hybridised primer of the duplex and occurrence of the template-dependent, primer extension reaction so as to incorporate the complementary terminator at the 3' end of the primer to form an extended primer such that the primer is extended by the complementary terminator if the particular nucleotide sequence is present in the template nucleic acid, no such extended primer being formed if the particular nucleotide sequence is not present in the template nucleic acid;
(f) removing labeled terminator not incorporated in the primer from the solid support; and
(g) detecting the presence or absence of a detectable marker at the 3' end of the immobilised primer from step (e) on the solid support, to determine the presence or absence of the particular nucleotide sequence in the nucleic acid of interest.

20. A method of typing a sample containing nucleic acids which comprises:

(a) determining the presence or absence of one or more particular nucleotide sequences, the presence or absence of each such nucleotide sequence being determined by a method as claimed in claim 19; and optionally
(b) identifying the nucleotide base or bases present at each of one or more specific positions, each such nucleotide base being identified using a method as claimed in any one of claims 1 to 18 and each such specific position being determined using a different primer.

Patentansprüche

1. Verfahren zur Bestimmung der Identität einer Nucleotidbase an einer spezifischen Position in einer interessierenden Nucleinsäure, aufweisend:

(a) alternativ:

(i) wenn die interessierende Nucleinsäure doppelsträngig ist, Behandeln einer die Nucleinsäure enthaltenden Probe, um eine Matrizen-Nucleinsäure-Probe mit ungepaarten Basen zu erhalten, die eine Matrize aus ungepaarten, sich über die spezifische Position erstreckenden Nucleotidbasen aufweist, oder

(ii) wenn die interessierende Nucleinsäure einzelsträngig ist, Erhalten einer Probe der Nucleinsäure, um als eine Matritzen-Nucleinsäure-Probe mit ungepaarten Basen zu dienen, die eine Matrize aus ungepaarten, sich über die spezifische Position erstreckenden Nucleotidbasen aufweist;

(b) Erhalten eines Oligonucleotid- Primers mit einer Nucleotidsequenz, die zur Hybridisierung mit der Matrizen-Nucleinsäure fähig ist, um eine Duplex zu bilden, in der die zu identifizierende Nucleotidbase dem 3'-Ende des Primers unmittelbar benachbart und stromab von ihm ist;

(c) Immobilisieren des Oligonucleotid-Primers auf einem festen Träger, um es dem immobiliserten Primer zu ermöglichen, mit der Matrizen-Nucleinsäure unter Bildung einer Duplex, in der die zu identifizierende Nucleotidbase dem 3'-Ende des Primers unmittelbar benachbart und stromab von ihm ist, zu hybridisieren;

(d) in Berührung Bringen des immobiliserten Oligonucleotid-Primers unter Hybridisierungsbedingungen mit der Matrizen-Probe mit ungepaarten Basen aus Schritt (a), um auf dem festen Träger eine Duplex zwischen dem immobilisierten Primer und der Matrizen-Nucleinsäure zu bilden, worin die zu identifizierende Nucleotidbase dem 3-Ende des Primers in der Duplex unmittelbar benachbart und stromab von ihm ist;

(e) in Berührung Bringen der Duplex aus Schritt (d) mit einer Reagens-Zusammensetzung, die einen wässerigen Träger und ein Gemisch von vier verschiedenen Terminatoren einer Nucleinsäure-Matrizen-abhängigen Primer-Extensionsreaktion aufweist, wobei jeder der Terminatoren zur spezifischen Beendigung der Extensionsreaktion in einer streng von der Identität der ungepaarten Nucleotidbase in der Matrize, die dem 3'-Ende des Primers unmittelbar benachbart und stromab von ihm ist, abhängigen Weise fähig ist, wobei einer der Terminatoren zu der zu identifizierenden Nucleotidbase komplementär ist und mindestens einer der Terminatoren mit einer nachweisbaren Markersubstanz markiert ist, unter Bedingungen, die eine Basenpaarung des in dem Reagens vorhandenen komplementären Terminators mit der zu identifizierenden Nucleotidbase und das Auftreten einer Matrizen-abhängigen Primer-Extensionsreaktion, um den komplementären Terminator am 3'-Ende des Primers unter Bildung eines verlängerten Primers dergestalt einzubauen, dass der Primer durch den komplementären Terminator verlängert wurde, erlauben;

(f) Entfernen von nicht in den Primer eingebautem markierten Terminator von dem festen Träger; und

(g) Identifizieren irgendeiner an dem 3'-Ende des immobilisierten verlängerten Primers aus Schritt (e) vorhandenen nachweisbaren Markersubstanz auf dem festen Träger, um die Identität der zu identifizierenden Nucleotidbase zu bestimmen.

2. Verfahren nach Anspruch 1, bei dem jeder der vier Terminatoren mit einer nachweismäßig verschiedenen nachweisbaren Markersubstanz markiert wird.

3. Verfahren nach Anspruch 1, bei dem der Oligonucleotid-Primer an einem von einer Mehrzahl getrennter Testplätze auf dem festen Träger immobilisiert wird.

4. Verfahren nach Anspruch 3, bei dem die Schritte (c) bis (g) viermal mit demselben Oligonucleotid-Primer aus Schritt (b) und mit derselben Matrizen-Probe mit ungepaarten Basen aus Schritt (a) an vier getrennten Testplätzen auf dem festen Träger wiederholt werden, wobei in Schritt (e) an jedem der vier Testplätze eine entsprechende der vier verschiedenen Reagens-Zusammensetzungen verwendet wird, wobei in jeder der vier Reagens-Zusammensetzungen nur einer der Terminatoren mit einer nachweisbaren Markersubstanz markiert ist, wobei ein unterschiedlicher der vier Terminatoren in jeder der vier verschiedenen Reagens-Zusammensetzungen markiert ist, so

dass das Identifizieren einer am 3'-Ende des immobilisierten verlängerten Primers von Schritt (e) vorhandenen nachweisbaren Markersubstanz an einem der vier Testplätze in Schritt (g) die Identität der zu identifizierenden Nucleotidbase bestimmt.

5. Verfahren nach einem der vorangehenden Ansprüche, bei dem in dem Reagens von Schritt (e) die Terminatoren ein Nucleotid oder ein Nucleotid-Analog aufweisen.

6. Verfahren nach Anspruch 5, bei dem die Terminatoren ein Dideoxynucleotid oder ein Arabinosid-triphosphat aufweisen.

7. Verfahren nach Anspruch 6, bei dem die Terminatoren eines oder mehrere der Triphosphate ddATP, ddCTP, ddGTP oder ddTTP aufweisen.

8. Verfahren zur Typisierung einer Nucleinsäuren enthaltenden Probe, aufweisend das Identifizieren der Nucleotidbase oder der Nucleotidbasen, die an jeder von einer oder von mehreren spezifischen Positionen vorhanden ist (sind), wobei jede derartige Nucleotidbase unter Verwendung eines Verfahrens, wie es in einem der Ansprüche 1 bis 7 beansprucht ist, identifiziert wird, und wobei jede derartige spezifische Position unter Verwendung eines unterschiedlichen Primers bestimmt wird, und bei dem die Identität jeder Nucleotidbase oder der Nucleotidbasen an jeder Position individuell bestimmt wird oder bei dem die Identitäten der Nucleotidbasen an verschiedenen Positionen gleichzeitig bestimmt werden.

9. Verfahren zur Identifizierung verschiedener Allele in einer Nucleinsäuren enthaltenden Probe, aufweisend das Identifizieren der Nucleotidbase oder der Nucleotidbasen, die an jeder von einer oder mehreren spezifischen Positionen vorhanden ist (sind), wobei jede derartige Nucleotidbase nach einem Verfahren, wie es in einem der Ansprüche 1 bis 7 beansprucht ist, identifiziert wird.

10. Verfahren zur Bestimmung des Genotyps eines Organismus an einem oder mehreren bestimmten Genorten, aufweisend:

    (a) Erhalten einer genomische DNA enthaltenden Probe von dem Organismus; und

    (b) Identifizieren der Nucleotidbase oder der Nucleotidbasen, die an jeder von einer oder mehreren spezifischen Positionen in interessierenden Nucleinsäuren vorhanden ist (sind), wobei die Base oder jede derartige Base unter Verwendung eines Verfahrens, wie es in einem der Ansprüche 1 bis 7 beansprucht ist, identifiziert wird, und dadurch Identifizieren von verschiedenen Alleleh, und dadurch wiederum Bestimmen des Genotyps des Organismus an einem bestimmten Genort oder mehreren bestimmten Genorten.

11. Verfahren nach einem der Ansprüche 1 bis 7, bei dem vor der Primer-Extensionsreaktion in Schritt (e) die Matrize an ihrem 3'-Ende durch die Hinzufügung eines Terminators zu dem 3'-Ende der Matrize mit einer Kappe versehen wurde, wobei der Terminator zur Beendigung einer Matrizen-abhängigen Primer-Extensionsreaktion fähig ist.

12. Verfahren nach einem der Ansprüche 1 bis 7, bei dem die Probe genomische DNA von einem Organismus, wozu eine Pflanze, ein Mikroorganismus, ein Virus, ein Vogel, ein Wirbeltier oder ein wirbelloses Tier, ein Säugetier, ein Mensch, ein Pferd, ein Hund, ein Rind, eine Katze, ein Schwein oder ein Schaf gehören, aufweist.

13. Verfahren nach einem der Ansprüche 1 bis 7, bei dem der Primer zu der bekannten Basensequenz, die der zu identifizierenden Base unmittelbar benachbart ist, komplementär ist.

14. Verfahren nach einem der Ansprüche 1 bis 7, bei dem der Primer unter Verwendung passender Denaturierungsbedingungen, wozu Wärme, Alkali, Formamid, Harnstoff, Glyoxal, Enzyme oder Kombinationen davon gehören, nach der Primer-Extensionsreaktion in Schritt (c) von der interessierenden Nucleinsäure abgetrennt wird.

15. Verfahren nach Anspruch 1, bei dem die mit nachweisbaren Markersubstanzen markierten Terminatoren mit nachweisbaren Markersubstanzen, die nicht radioaktiv sind, markiert werden.

16. Verfahren nach Anspruch 15, bei dem der Schritt (g) des Identifizierens irgendeiner nachweisbaren Markersubstanz an dem 3'-Ende des immobilisierten verlängerten Primers durch visuelle oder optische Mittel ausgeführt wird.

17. Verfahren nach Anspruch 16, bei dem der feste Träger für ein Nachweisverfahren eines optischen Signals geeignet ist.

18. Verfahren nach Anspruch 17, bei dem der feste Träger für einen optischen Signalnachweis unter Verwendung eines automatischen Plattenlesegeräts geeignet ist.

19. Verfahren zu Bestimmung der Anwesenheit oder Abwesenheit einer bestimmten Nucleotidsequenz in einer interessierenden Nucleinsäure, aufweisend:

(a) alternativ:

(i) wenn die interessierende Nucleinsäure doppelsträngig ist, Behandeln einer die Nucleinsäure enthaltenden Probe, um eine Matrizen-Nucleinsäure-Probe mit ungepaarten Basen zu erhalten, die eine Matrize aus ungepaarten Nucleotidbasen aufweist, oder

(ii) wenn die interessierende Nucleinsäure einzelsträngig ist, Erhalten einer Probe der Nucleinsäure, um als eine Matrizen-Nucleinsäure-Probe mit ungepaarten Basen, die eine Matrize aus ungepaarten Nucleotidbasen aufweist, zu dienen;

(b) Erhalten eines Oligonucleotid-Primers mit einer Nucleotidsequenz, die zur Hybridisierung mit der bestimmten Nucleotidsequenz fähig ist, wenn die bestimmte Nucleotidsequenz in der Matrizen-Nucleinsäure vorhanden ist, um eine Primer-verlängerbare Duplex zwischen dem Primer und der bestimmten Nucleotidsequenz zu bilden, so dass der hybridisierte Primer an einem 3'-Ende durch eine Nucleinsäure-Matrizen-abhängige Primer-Extensionsreaktion verlängerbar ist;

(c) Immobilisieren des Oligonucleotid-Primers an einem festen Träger, um es zu ermöglichen, dass der immobilisierte Primer mit der Matrizen-Nucleinsäure unter Bildung einer Primer-verlängerbaren Duplex hybridisiert, wenn die bestimmte Nucleotidsequenz in der Matrizen-Nucleinsäure vorhanden ist, so dass der hybridisierte Primer an dem 3'-Ende durch eine Nucleinsäure-Matrizen-abhängige Primer-Extensionsreaktion verlängerbar ist;

(d) in Berührung Bringen des immobiliserten Oligonucleotid-Primers unter Hybridisierungsbedingungen mit einer Matrizen-Probe mit ungepaarten Basen aus Schritt (a), um auf dem festen Träger eine Primer-verlängerbare Duplex zwischen dem immobilisierten Primer und der Matrizen-Nucleinsäure zu bilden, wenn die bestimmte Nucleotidsequenz in der Matrizen-Nucleinsäure vorhanden ist, so dass der hybridisierte Primer an dem 3'-Ende durch eine Nucleinsäure-Matrizen-abhängige Primer-Extensionsreaktion verlängerbar ist;

(e) in Berührung Bringen der Primer-verlängerbaren Duplex aus Schritt (d), falls vorhanden, mit einer Reagens-Zusammensetzung, die einen wässerigen Träger und ein Gemisch von vier verschiedenen Terminatoren einer Nucleinsäure-Matrizen-abhängigen Primer-Extensionsreaktion aufweist, wobei mindestens einer der Terminatoren mit einer nachweisbaren Markersubstanz markiert wird, wobei jeder der Teminatoren zur spezifischen Beendigung der Primer-Extensionsreaktion in einer von der Identität einer ungepaarten Nucleotidbase in einer Matrize, die dem 3'-Ende eines Primers, der an die Matrize so hybridisiert ist, dass der hybridisierte Primer an einem 3'-Ende durch die Primer-Extensionsreaktion verlängerbar ist, unmittelbar benachbart und stromab von ihm ist, abhängigen Weise fähig ist, wobei die Primer-verlängerbare Duplex, falls vorhanden, mit der Reagens-Zusammensetzung unter Bedingungen in Berührung gebracht wird, die eine Basenpaarung des in dem Reagens vorhandenen komplementären Terminators mit einer Nucleotidbase in der Matrizen-Nucleinsäure der Duplex, die dem 3'-Ende des hybridisierten Primers der Duplex unmittelbar benachbart und stromab von ihm ist, und das Auftreten der Matrizen-abhängigen Primer-Extensionsreaktion, um den komplementären Terminätor an dem 3'-Ende des Primers einzubauen, um einen verlängerten Primer zu bilden, so dass der Primer durch den komplementären Terminator verlängert wird, wenn die bestimmte Nucleotidsequenz in der Matrizen-Nucleinsäure vorhanden ist, wobei kein derartiger verlängerter Primer gebildet wird, wenn die bestimmte Nucleotidsequenz in der Matrizen- Nucleinsäure nicht vorhanden ist, erlauben;

(f) Entfernen von nicht in den Primer eingebautem markiertem Terminator von dem festen Träger; und

(g) Nachweisen der Anwesenheit oder Abwesenheit einer nachweisbaren Markierungssubstanz an dem 3'-Ende des immobiliserten Primers aus Schritt (e) auf dem festen Träger, um die Anwesenheit oder Abwesenheit

der bestimmten Nucleotidsequenz in der interessierenden Nucleinsäure zu bestimmen.

20. Verfahren zur Typisierung einer Nucleinsäuren enthaltenden Probe, aufweisend:

(a) Bestimmen der Anwesenheit oder Abwesenheit einer oder mehrerer bestimmter Nucleotidsequenzen, wobei die Anwesenheit oder Abwesenheit jeder derartigen Nucleotidsequenz durch ein Verfahren, wie es in Anspruch 19 beansprucht wird, bestimmt wird; und gewünschtenfalls

(b) Identifizieren der Nucleotidbase oder der Nucleotidbasen, die an jeder von einer oder mehreren spezifischen Positionen vorhanden ist (sind), wobei jede derartige Nucleotidbase unter Verwendung eines Verfahrens, wie es in einem der Ansprüche 1 bis 18 beansprucht wird, identifiziert wird, und wobei jede derartige spezifische Position unter Verwendung eines unterschiedlichen Primers bestimmt wird.

Revendications

1. Procédé de détermination de l'identité d'une base nucléotidique à une position spécifique dans un acide nucléique d'intérêt qui comprend :

(a) à titre d'alternative :

(i) si l'acide nucléique d'intérêt est double brin, le traitement d'un échantillon contenant l'acide nucléique de manière à obtenir un échantillon d'acide nucléique de matrice à bases non appariées comprenant une matrice de bases nucléotidiques non appariées s'étendant sur la position spécifique, ou
(ii) si l'acide nucléique d'intérêt est simple brin, l'obtention d'un échantillon de l'acide nucléique pour servir d'échantillon d'acide nucléique de matrice à bases non appariées comprenant une matrice de bases nucléotidiques non appariées s'étendant sur la position spécifique ;

(b) l'obtention d'un amorce oligonucléotidique ayant une séquence nucléotidique capable de s'hybrider avec l'acide nucléique de matrice pour former un duplex où la base nucléotidique à identifier est immédiatement adjacente et en aval de l'extrémité 3' de l'amorce ;
(c) l'immobilisation de l'amorce oligonucléotidique sur un support solide de manière à permettre à l'amorce immobilisée de s'hybrider avec l'acide nucléotidique de matrice pour former un duplex où la base nucléotidique à identifier est immédiatement adjacente et en aval de l'extrémité 3' de l'amorce
(d) la mise en contact de l'amorce nucléotidique immobilisée dans des conditions d'hybridation avec l'échantillon de matrice à bases non appariées de l'étape (a) pour former sur le support solide un duplex entre l'amorce immobilisée et l'acide nucléique de matrice quand la base nucléotidique à identifier est immédiatement adjacente et en aval de l'extrémité 3' de l'amorce dans le duplex ;
(e) la mise en contact du duplex de l'étape (d) avec une composition de réactifs qui comprend un support aqueux et un mélange de quatre terminateurs différents d'une réaction d'extension d'amorce dépendant de matrice d'acide nucléique, chacun des terminateurs étant capable de terminer spécifiquement la réaction d'extension d'une manière strictement dépendante de l'identité de la base nucléotidique non appariée dans la matrice immédiatement adjacente et en aval de l'extrémité 3' d'une amorce, où l'un des terminateurs est complémentaire de la base nucléotidique à identifier et au moins l'un des terminateurs est marqué avec un marqueur détectable, dans des conditions permettant l'appariement de bases du terminateur complémentaire présent dans le réactif avec la base nucléotidique, à identifier et la survenue d'une réaction d'extension d'amorce dépendante de matrice de manière à incorporer le terminateur complémentaire à l'extrémité 3' de l'amorce pour former une amorce étendue de telle sorte que l'amorce a été étendue par le terminateur complémentaire.
(f) le retrait du terminateur marqué non incorporé dans l'amorce du support solide ; et
(g) l'identification de tout marqueur détectable présent à l'extrémité 3' de l'amorce étendue immobilisée de l'étape (e) sur le support solide, pour déterminer l'identité de la base nucléotidique à identifier.

2. Procédé selon la revendication 1, où chacun des quatre terminateurs est marqué avec un marqueur détectable différent d'une manière détectable.

3. Procédé selon la revendication 1, où l'amorce oligonucléotidique est immobilisée à un emplacement d'une pluralité d'emplacements de test séparés sur le support solide.

4. Procédé selon la revendication 3, où les étapes (c) à (g) sont répétées quatre fois avec la même amorce oligonucléotidique provenant de l'étape (b) et le même échantillon de matrice à bases non appariées provenant de l'étape (a) à quatre emplacements de test séparés sur le support solide, une composition du réactif parmi quatre compositions de réactifs différentes étant utilisée dans l'étape (e) à chacun des quatre emplacements de test, dans chacune des quatre compositions de réactifs seulement l'un des terminateurs étant marqué avec un marqueur détectable, un terminateur différent parmi les quatre terminateurs étant marqué dans chacune des quatre compositions de réactifs différentes, de sorte que l'identification d'un marqueur détectable présent à l'extrémité 3' de l'amorce étendue immobilisée provenant de l'étape (e) à l'un des quatre emplacements de test de l'étape (g) détermine l'identité de la base nucléotidique à identifier.

5. Procédé selon l'une quelconque des revendications précédentes, où, dans le réactif de l'étape (e), les terminateurs comprennent un nucléotide ou un analogue de nucléotide.

6. Procédé selon, la revendication 5, où les terminateurs comprennent un didésoxynucléotide ou un arabinoside triphosphate.

7. Procédé selon la revendication 6, où les terminateurs comprennent un ou plusieurs parmi ddATP, ddCTP, ddGTP ou ddT7-P.

8. Procédé de typage d'un échantillon contenant des acides nucléiques qui comprend l'identification de la base nucléotidique ou des bases nucléotidiques présentes à chaque position parmi une ou plusieurs positions spécifiques, chacune de ces bases nucléotidiques étant identifiée au moyen d'un procédé selon l'une quelconque des revendications 1 à 7 et chacune des positions spécifiques étant déterminée au moyen d'une amorce différente et où l'identité de chaque base nucléotidique ou des bases nucléotidiques à chaque position est déterminée individuellement ou bien où l'identité des bases nucléotidiques à différentes positions sont déterminées simultanément.

9. Procédé pour identifier des allèles différents dans un échantillon contenant des acides nucléiques qui comprend l'identification de la base nucléotidique ou des bases nucléotidiques présentes à chaque position parmi une ou plusieurs positions spécifiques, chacune des bases nucléotidiques étant identifiée par un procédé selon l'une quelconque des revendications 1 à 7.

10. Procédé pour déterminer le génotype d'un organisme à un ou plusieurs locus génétiques particuliers qui comprend :

    (a) l'obtention à partir de l'organisme d'un échantillon contenant de l'ADN génomique ; et
    (b) l'identification de la base nucléotidique ou des bases nucléotidiques présentes à chacune d'une ou plusieurs positions spécifiques dans les acides nucléiques d'intérêt, chaque base ou les bases étant identifiées au moyen d'un procédé selon l'une quelconque des revendications 1 à 7 pour identifier différents allèles et ainsi pour déterminer ensuite le génotype de l'organisme à un ou plusieurs locus génétiques particuliers.

11. Procédé selon l'une quelconque des revendications 1 à 7, où, avant la réaction d'extension d'amorce dans l'étape (e), la matrice a été coiffée à son extrémité 3' par addition d'un terminateur à l'extrémité 3' de la matrice, ledit terminateur étant capable de terminer une réaction d'extension d'amorce dépendante de matrice.

12. Procédé selon l'une quelconque des revendications 1 à 7, où l'échantillon comprend de l'ADN génomique provenant d'un organisme, incluant une plante, un microorganisme, un virus, un oiseau, un vertébré ou un invertébré, un mammifère, un être humain, un cheval, un chien, une vache, un chat, un porc ou un mouton.

13. Procédé selon l'une quelconque des revendications 1 à 7, où l'amorce est complémentaire de la séquence de bases connue immédiatement adjacente à la base à identifier.

14. Procédé selon l'une quelconque des revendications 1 à 7, où l'amorce est séparée de l'acide nucléique d'intérêt après la réaction d'extension d'amorce dans l'étape (c) au moyen de conditions dénaturantes appropriées, incluant la chaleur, un alcali, le formamide, l'urée, le glyoxal, des enzymes ou des combinaisons de celles-ci.

15. Procédé selon la revendication 1, où les terminateurs marqués avec des marqueurs détectables sont marqués avec des marqueurs détectables qui sont non radioactifs.

16. Procédé selon la revendication 15, où l'étape (g) d'identification d'un marqueur détectable quelconque à l'extrémité 3' de l'amorce étendue immobilisée est réalisée par des moyens visuels ou optiques

17. Procédé selon la revendication 16, où le support solide est adapté pour un procédé de détection de signal optique.

18. Procédé selon la revendication 17, où le support solide est adapté pour la détection de signal optique au moyen d'un lecteur de plaques automatique.

19. Procédé de détermination de la présence ou de l'absence d'une séquence nucléotidique particulière dans un acide nucléique d'intérêt qui comprend :

(a) à titre d'alternative :

(i) si J'acide nucléique d'intérêt est double brin, le traitement d'un échantillon contenant l'acide nucléique de manière à obtenir un échantillon d'acide nucléique de matrice à bases non appariées comprenant une matrice de bases nucléotidiques non appariées, ou
(ii) si l'acide nucléique d'intérêt est simple brin, l'obtention d'un échantillon de l'acide nucléique pour servir d'échantillon d'acide nucléique de matrice à bases non appariées comprenant une matrice de bases nucléotidiques non appariées;

(b) l'obtention d'un amorce oligonucléotidique ayant une séquence nucléotidique capable de s'hybrider avec la séquence nucléotidique particulière si la séquence nucléotidique particulière est présente dans l'acide nucléique de matrice pour former un duplex extensible par amorce entre l'amorce et la séquence nucléotidique particulière de telle sorte que l'amorce hybridée est extensible à une extrémité 3' par une réaction d'extension d'amorce dépendante de matrice d'acide nucléique ;
(c) l'immobilisation de l'amorce oligonucléotidique sur un support solide de manière à permettre à l'amorce immobilisée de s'hybrider avec l'acide nucléique de matrice pour former un duplex extensible par amorce si la séquence nucléotidique particulière est présente dans l'acide nucléique de matrice de telle sorte que l'amorce hybridé est extensible à l'extrémité 3' par une réaction d'extension d'amorce dépendante de matrice d'acide nucléique ;
(d) la mise en contact de l'amorce nucléotidique immobilisée dans des conditions d'hybridation avec un échantillon de matrice à bases non appariées provenant de l'étape (a) pour former sur le support solide un duplex extensible par amorce entre l'amorce immobilisée et l'acide nucléique de matrice si la séquence nucléotidlque particulière est présente dans l'acide nucléique de matrice de telle sorte que l'amorce hybridée est extensible à l'extrémité 3' par une réaction d'extension d'amorce dépendante de matrice d'acide nucléique
(e) la mise en contact du duplex extensible par amorce provenant de l'étape (d), s'il y en a, avec une composition de réactifs qui comprend un support aqueux et un mélange de quatre terminateurs différents d'une réaction d'extension d'amorce dépendante de matrice d'acide nucléique, au moins l'un des terminateurs étant marqué avec un marqueur détectable, chacun des terminateurs étant capable de terminer spécifiquement la réaction d'extension d'amorce d'une manière dépendante de l'identité d'une basé nucléotidique non appariée dans une matrice immédiatement adjacente et en aval de l'extrémité 3' d'une amorce hybridée à la matrice de telle sorte que la matrice hybridée est extensible à une extrémité 3' par la réaction d'extension d'amorce, le duplex extensible par amorce, s'il y en a, étant mis en contact avec la composition de réactifs dans des conditions permettant un appariement de bases d'un terminateur complémentaire présent dans le réactif avec une base nucléotidique dans l'acide nucléique de matrice du duplex immédiatement adjacent et en aval de l'extrémité 3' de l'amorce hybridée du duplex et la survenue de la réaction d'extension d'amorce dépendante de matrice de manière à incorporer le terminateur complémentaire à l'extrémité 3' de l'amorce pour former une amorce étendue de telle sorte que l'amorce est étendue par le terminateur complémentaire si la séquence nucléotidique particulière est présente dans l'acide nucléique de matrice, aucune amorce étendue de ce type n'étant formée si la séquence nucléotidique particulière n'est pas présente dans l'acide nucléique de matrice ;
(f) le retrait du terminateur marqué non incorporé dans l'amorce du support solide ; et
(g) la détection de la présence ou de l'absence d'un marqueur détectable à l'extrémité 3' de l'amorce immobilisée provenant de l'étape (e) sur le support solide, pour déterminer la présence ou l'absence de la séquence nucléotidique particulière dans l'acide nucléique d'intérêt.

20. Procédé de typage d'un échantillon contenant des acides nucléiques qui comprend :

(a) la détermination de la présence ou de l'absence d'une ou plusieurs séquences nucléotidiques particulières,

la présence ou l'absence de chaque séquence nucléotidique de ce type étant déterminée par un procédé selon la revendication 19 ; et éventuellement

(b) l'identification de la base nucléotidique ou des bases nucléotidiques présentes à chacune d'une ou plusieurs positions spécifiques, chaque base nucléotidique de ce type étant identifiée au moyen d'un procédé selon l'une quelconque des revendications 1 à 18 et chaque position spécifique de ce type étant déterminée au moyen d'une amorce différente.

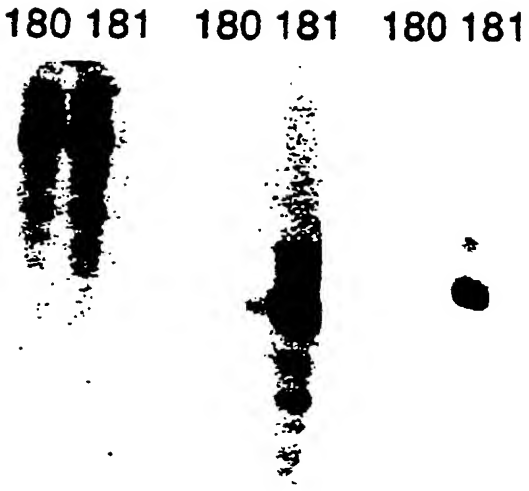


FIG. 1A FIG. 1B FIG. 1C

I. Amplification primers

TGL 105: 5'-TTCTTCTTGCATCTATGTTCG-3'

TGL 106: 5'-TTAAGCACCACCACAGGTCCT-3'

II. Polymorphism detection primers

TGL 182: 5'-GCCTTGGCGTTGTAGAA-3'

TGL 166: 5'-AGAGAAACAATTTCAAG-3'

III. Target sequence

```
5' ...TTTCTTCTTG CATCTATGTT CGTTTTTTCT ATTGCTACAA     40
       TGL 105 ----->

   ATGCCTATGC ACGGCCTGAC TTCTGCCTAG AGCCTCCATA     80

   TACGGGTCCC TGCAAGGCCA GAATTATCAG ATA[C]/TTTCTAC  120

   AACGCCAAGG CTGGGCTCTG CCAGACCTTT GTATATGGTG     160
   <----- TGL 182

   GCTGCAGAGC TAAGAGAAAC AATTTCAAG[A]/G GCGCAGAGGA  200
                TGL 166 ----->

   CTGCATGAGG ACCTGTGGTG GTGCTTAAGG GCCCCGGGAA..3'240
                     <----- TGL 106
```

IV. Polymorphisms

Plasmid	Nucleotide 114	Nucleotide 190
p183	C	A
p624	T	A
p814	C	G

FIGURE 2

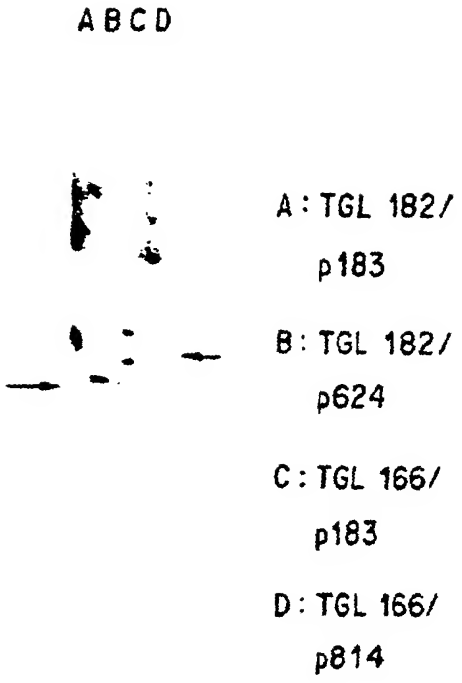


FIG. 3

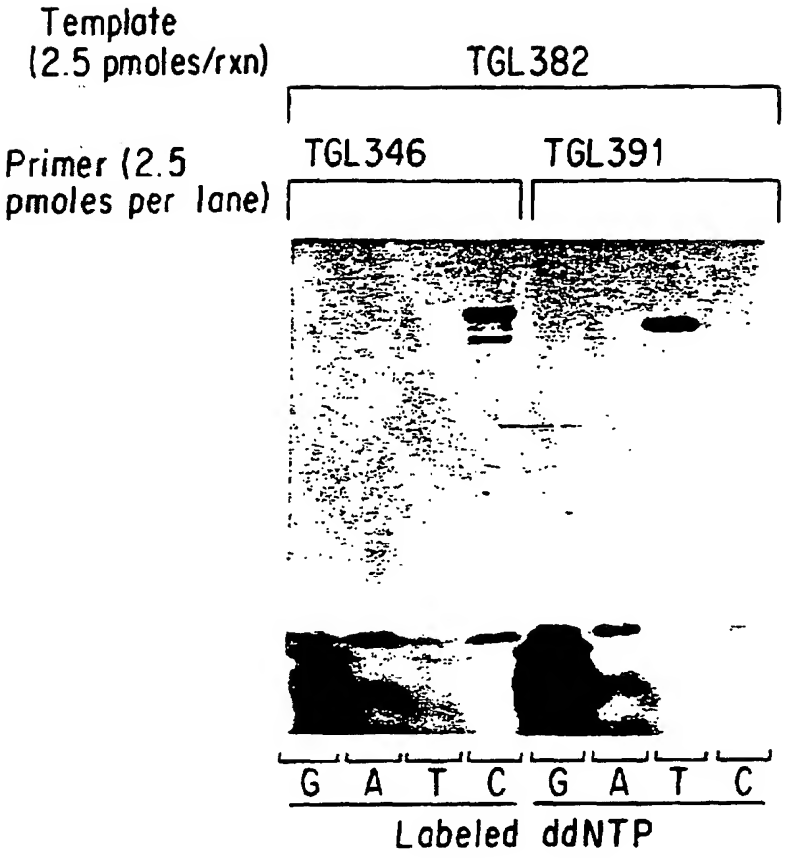

Template
(2.5 pmoles/rxn)
TGL382
Primer (2.5
pmoles per lane)
TGL346
TGL391
G A T C G A T C
Labeled ddNTP

FIG. 4

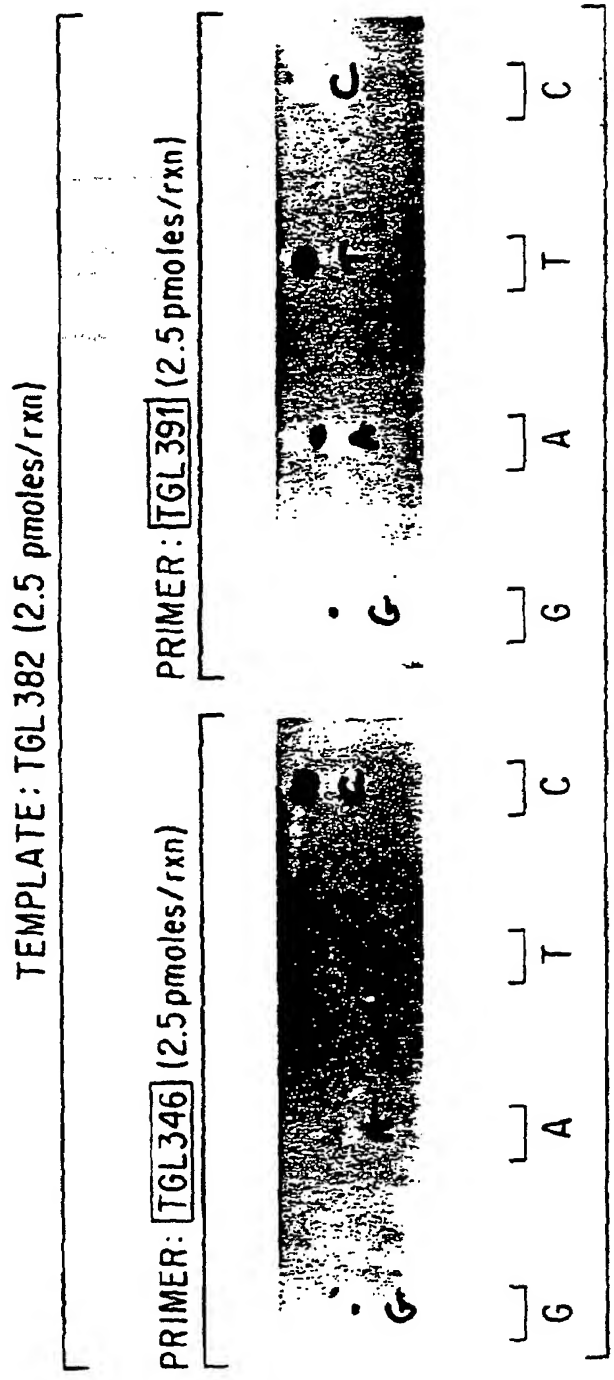

TEMPLATE: TGL382 (2.5 pmoles/rxn)
PRIMER: TGL346 (2.5 pmoles/rxn)
PRIMER: TGL391 (2.5 pmoles/rxn)
G A T C G A T C
LABELED ddNTP

FIG. 5

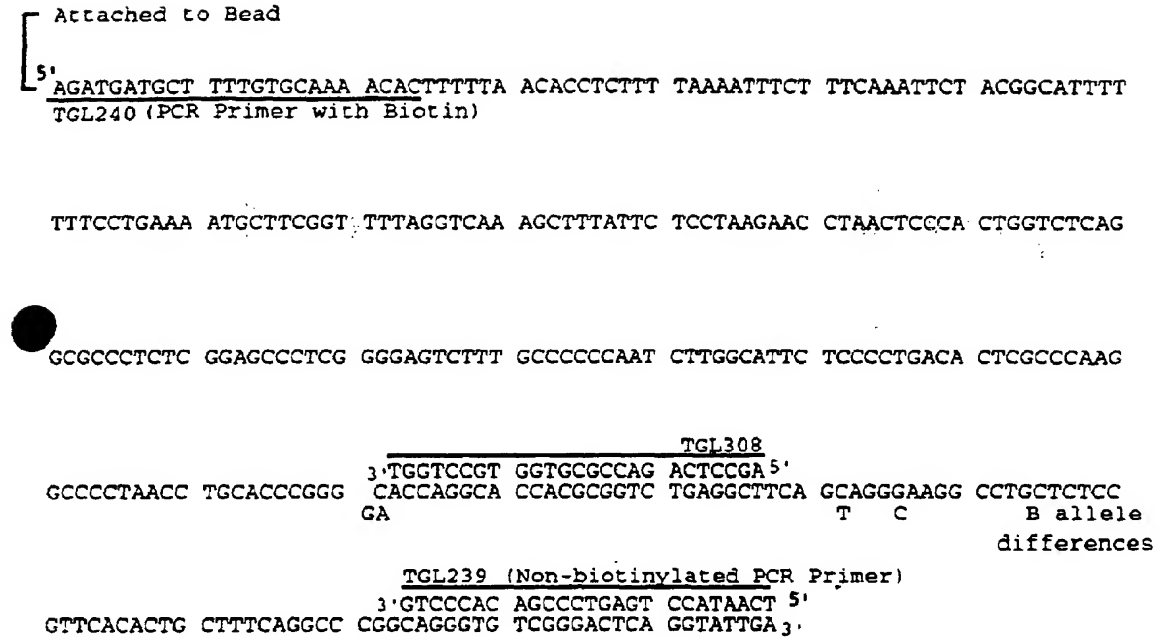

Attached to Bead
5'
AGATGATGCT TTTGTGCAAA ACACTTTTTA ACACCTCTTT TAAAATTTCT TTCAAATTCT ACGGCATTTT
TGL240 (PCR Primer with Biotin)
TTTCCTGAAA ATGCTTCGGT TTTAGGTCAA AGCTTTATTC TCCTAAGAAC CTAACTCCCA CTGGTCTCAG
GCGCCCTCTC GGAGCCCTCG GGGAGTCTTT GCCCCCCAAT CTTGGCATTC TCCCCTGACA CTCGCCCAAG
TGL308
3'TGGTCCGT GGTGCGCCAG ACTCCGA 5'
GCCCCTAACC TGCACCCGGG CACCAGGCA CCACGCGGTC TGAGGCTTCA GCAGGGAAGG CCTGCTCTCC
GA T C
B allele
differences
TGL239 (Non-biotinylated PCR Primer)
3'GTCCCAC AGCCCTGAGT CCATAACT 5'
GTTCACACTG CTTTCAGGCC CGGCAGGGTG TCGGGACTCA GGTATTGA 3'

FIGURE 6

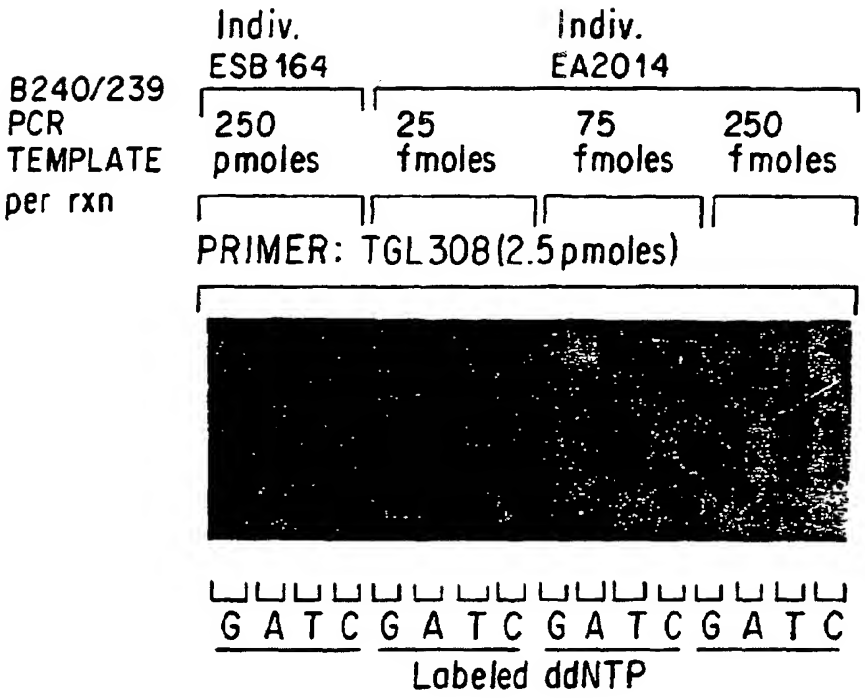

Indiv.
ESB164
Indiv.
EA2014
B240/239
PCR
TEMPLATE
per rxn
250
pmoles
25
fmoles
75
fmoles
250
fmoles
PRIMER: TGL308 (2.5 pmoles)
G A T C G A T C G A T C G A T C
Labeled ddNTP

FIG. 7

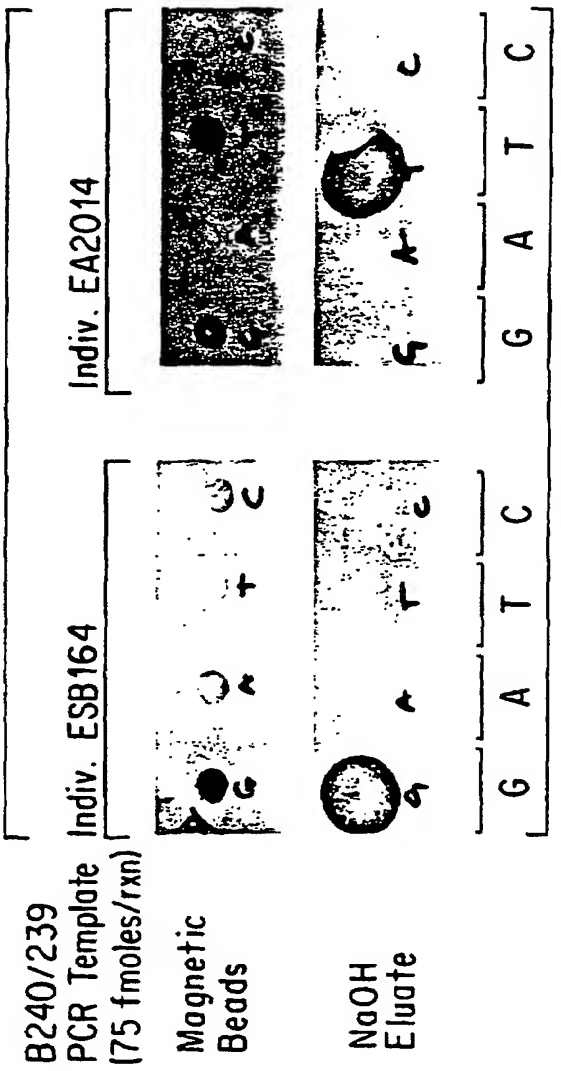

PRIMER: TGL308(2.5 pmoles/rxn)
B240/239
PCR Template
(75 fmoles/rxn)
Indiv. ESB164
Indiv. EA2014
Magnetic
Beads
NaOH
Eluate
G A T C
G A T C
Labeled ddNTP

FIG. 8

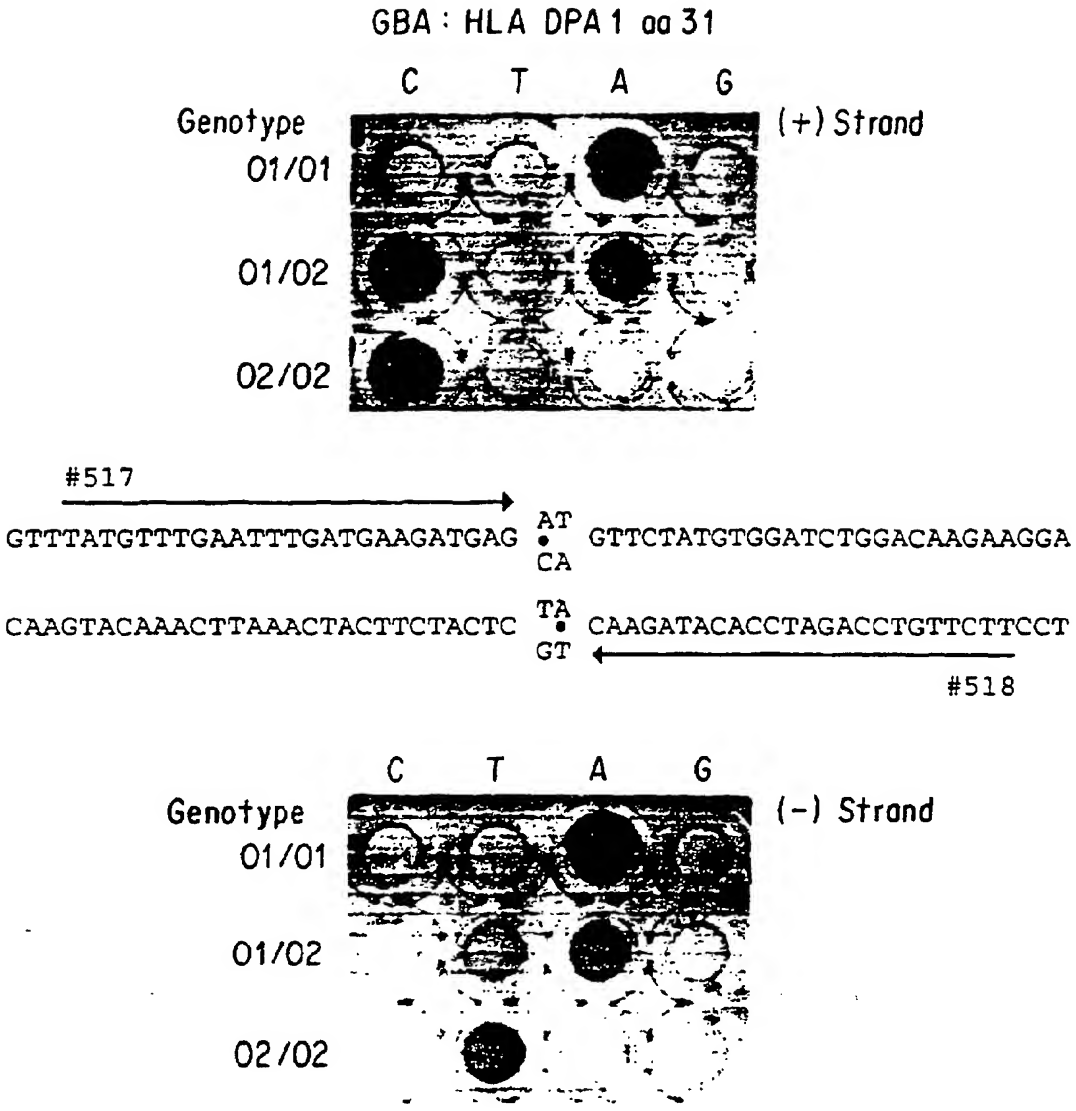

GBA : HLA DPA 1 aa 31
C T A G
Genotype
01/01
01/02
02/02
(+) Strand
#517
GTTTATGTTTGAATTTGATGAAGATGAG AT CA GTTCTATGTGGATCTGGACAAGAAGGA
CAAGTACAAACTTAAACTACTTCTACTC TA GT CAAGATACACCTAGACCTGTTCTTCCT
#518
C T A G
Genotype
01/01
01/02
02/02
(-) Strand

FIG. 9

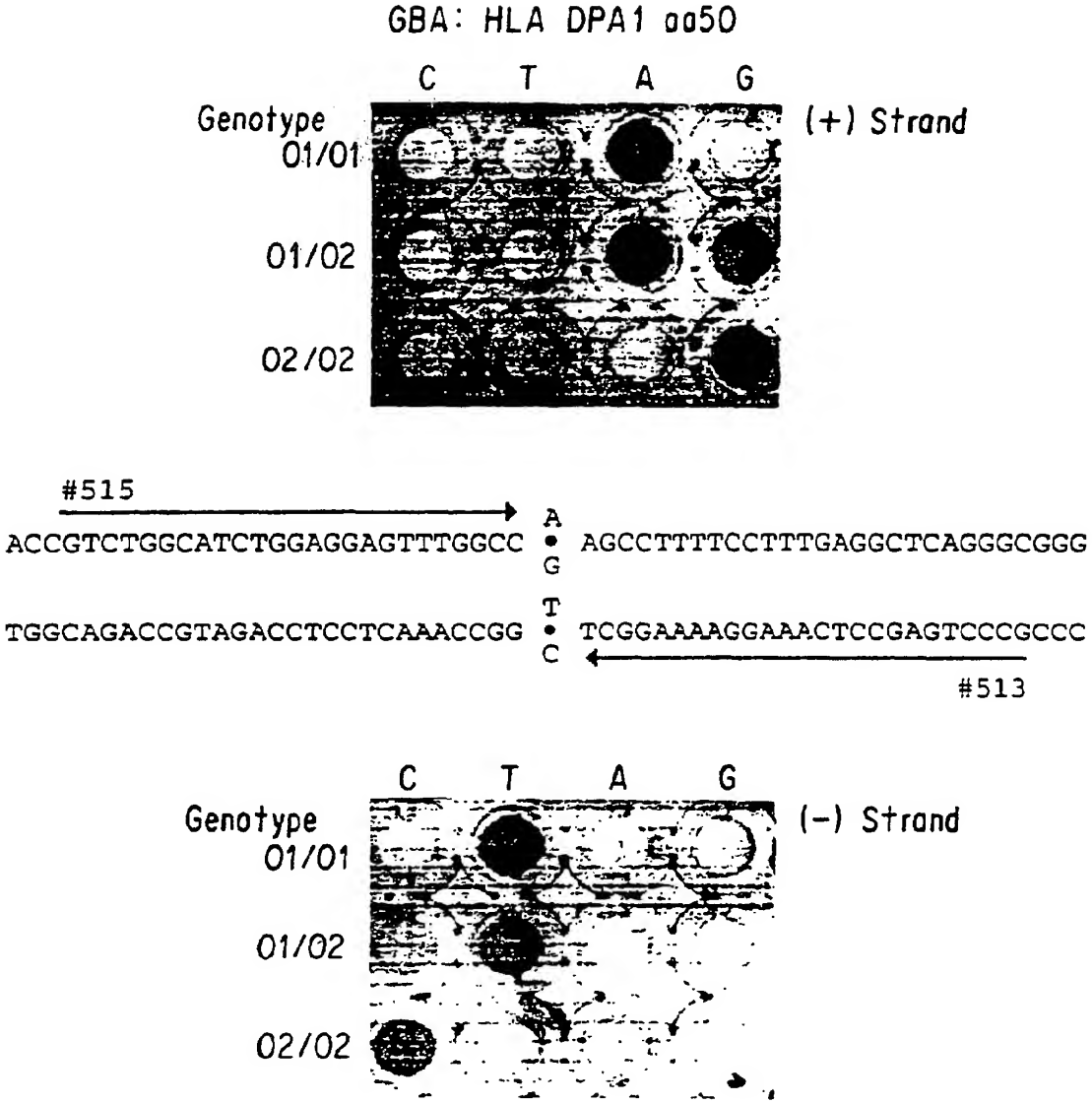

GBA: HLA DPA1 aa50
C T A G
Genotype
01/01
01/02
02/02
(+) Strand
#515
ACCGTCTGGCATCTGGAGGAGTTTGGCC
A
G
AGCCTTTTCCTTTGAGGCTCAGGGCGGG
TGGCAGACCGTAGACCTCCTCAAACCGG
T
C
TCGGAAAAGGAAACTCCGAGTCCCGCCC
#513
C T A G
Genotype
01/01
01/02
02/02
(-) Strand

FIG. 10

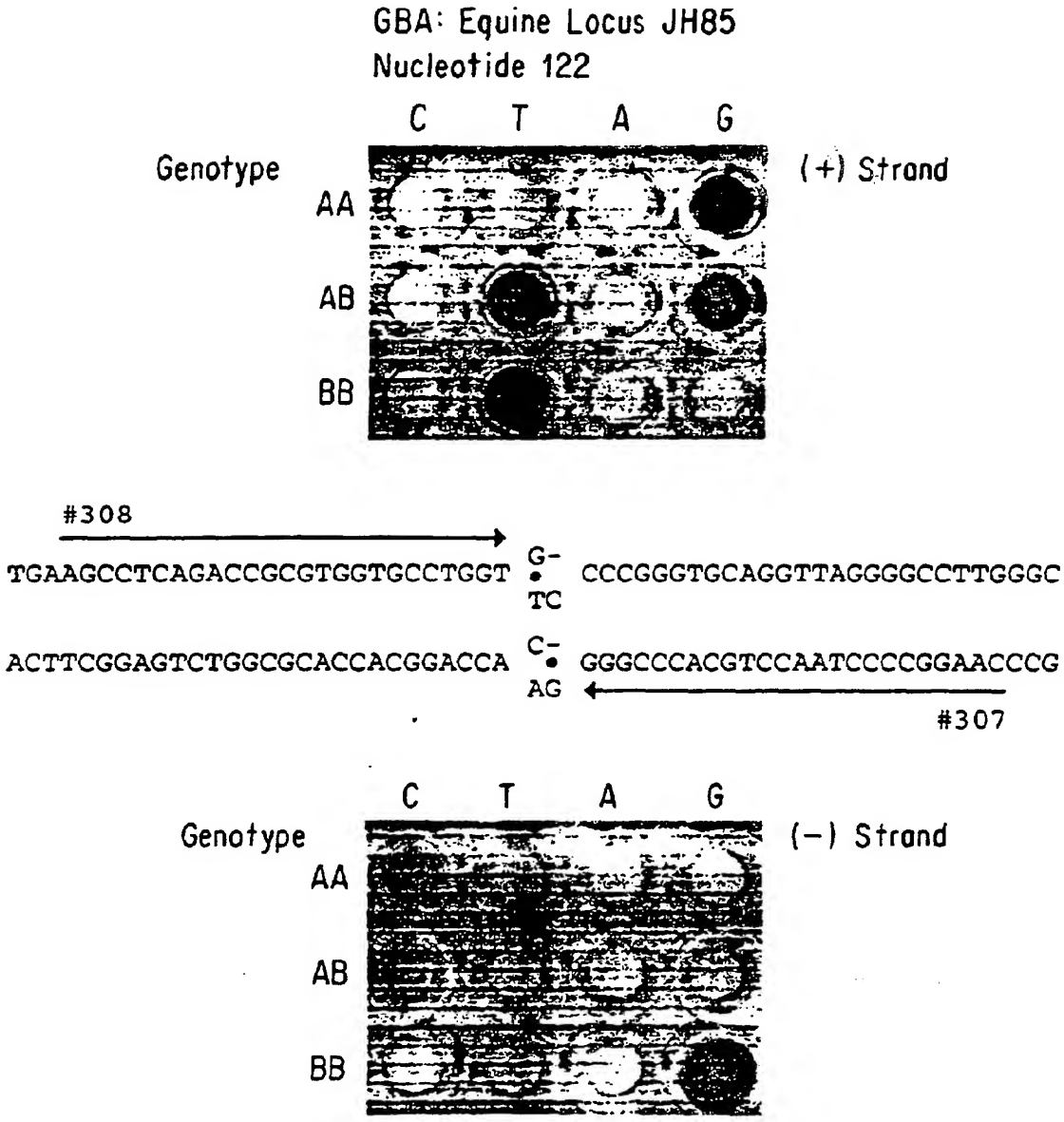
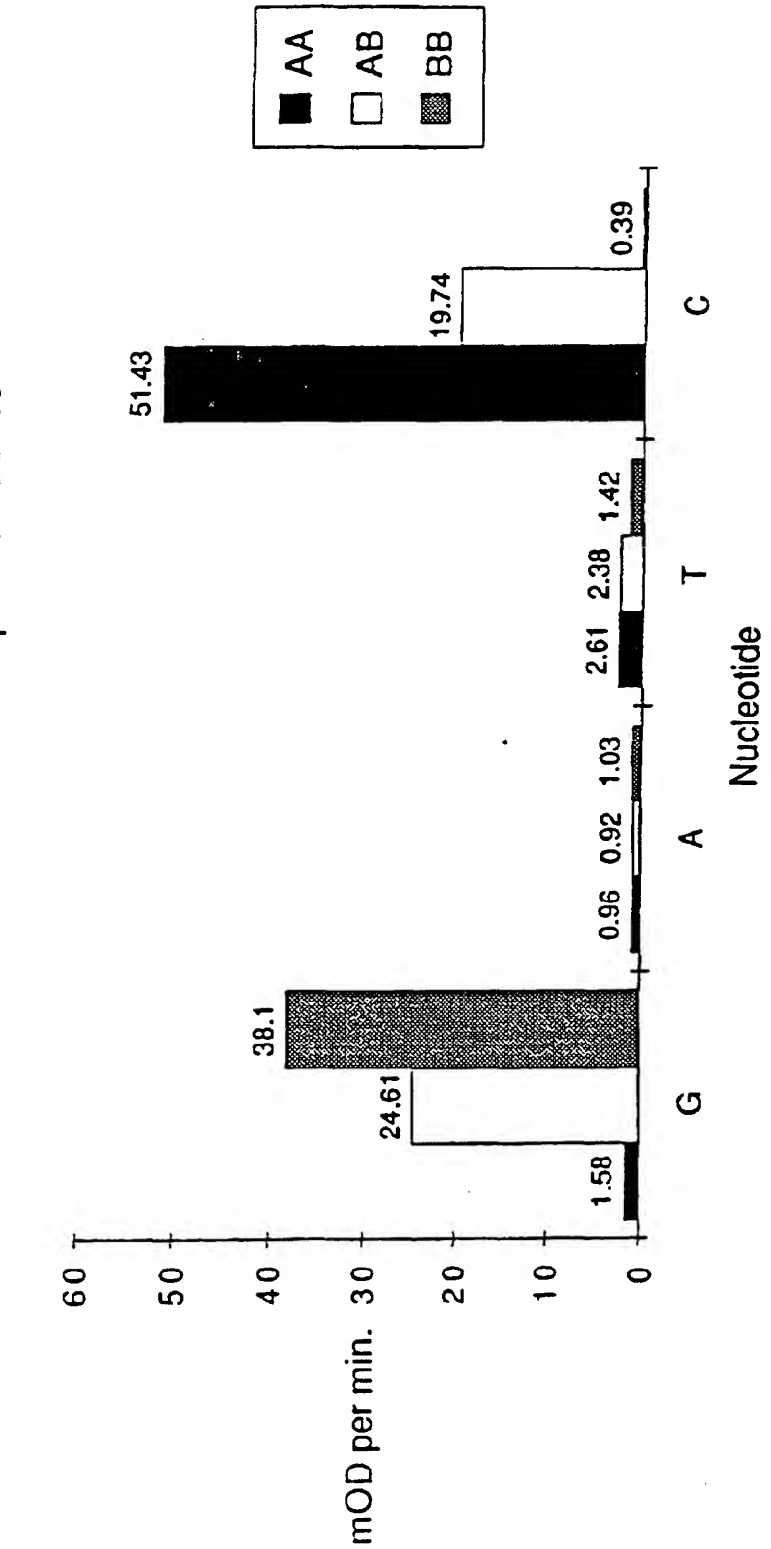

GBA: Equine Locus JH85
Nucleotide 122
C T A G
Genotype
AA
AB
BB
(+) Strand
#308
TGAAGCCTCAGACCGCGTGGTGCCTGGT
G-
•
TC
CCCGGGTGCAGGTTAGGGGCCTTGGGC
ACTTCGGAGTCTGGCGCACCACGGACCA
C-
•
AG
GGGCCCACGTCCAATCCCCGGAACCCG
#307
C T A G
Genotype
AA
AB
BB
(−) Strand

FIG. 11

FIGURE 12


QUANTITATIVE GBA: Equine locus JH85
AA
AB
BB
mOD per min.
0
10
20
30
40
50
60
1.58
24.61
38.1
0.96
0.92
1.03
2.61
2.38
1.42
51.43
19.74
0.39
G
A
T
C
Nucleotide